Investigating the Oxidoreductase Activity of Members of the Chloride Intracellular Ion Channel Protein Family

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Certificate of Original Authorship

I, Hala Mishaal Ali, certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

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Publications

Publications arising from this thesis

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Conference Presentations

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1- Hala M Ali, Khondker R Hossain, Heba Al Khamici, Lele Jiang, Stella M Valenzuela (**2016**) Members of the Chloride Intracellular Ion Channel Protein Family Demonstrate Cell Protective Effect Against Oxidative Stress New Horizons, Sydney, Australia.

2- Hala M Ali, Khondker R Hossain, Stella M Valenzuela (**2017**) Members of the Chloride Intracellular Ion Channel Protein Family Catalyse Protein Deglutathionylation. New Horizons, Sydney, Australia.

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Oral presentations

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List of Abbreviations

AsA	Ascorbic Acid
Å	Angstrom
Arg	Arginine amino acid
Asn	Asparagine amino acid
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid assay
BSA	bovine serum albumin
°C	centigrade
CaCCs	Calcium activated Chloride channel
CaCl2	calcium chloride
CAFs	Cancer-Associated Fibroblast secretome
Car	Carbenicillin
CDCs	Cholesterol-Dependent Cytolysins
C-domain	Carboxy terminal domain
cDNA	complementary Deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO-k1	Chinese Hamster Ovary cells
Chol	Cholesterol
CIC	Chloride Ion Channel
CLIC	Chloride Intracellular Ion Channel
Cl^-	Chloride ion
CLIC (WT)	Chloride Intracellular Ion Channel protein (wild type)
Cys	Cysteine
CV	column volumes
COPD	Chronic obstructive pulmonary disease
Da	Dalton
DDT	Dithiotheritol
DHA	Dehydroascorbate

DHAR	Dehydroascorbic acid reductase
DMSO	Dimethyl sulfoxide
Dm CLIC	*Drosophila melanogaster* CLIC protein
DNA	Deoxyribonucleotides acid
DIDS	4, 4'Di isothiocyano-2, 2'-stilbenedisulfonic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
E-coli	*Escherichia coli*
EPR	Electron Paramagnetic Resonance
ER	Endoplasmic Reticulum
ERK7	Extracellular signal-regulated kinase 7
EXC	Excretory Canal abnormality
EXL	EXC4-like
FRET	Fluorescence Resonance Energy Transfer
3D	three dimensions
GABA	Gamma-Amino Butyric Acid
Glu	Glutamic amino acid
Grx	Glutaredoxin
GR	glutathione reductase
GSH	Reduced glutathione
G-site	Glutathione binding site
GST	Glutathione S-transferase
HEDS	2-hydroxyethyl disulphide
His	Histidine
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H2O	water
IAA	Indanyloxyacetic acid
IPTG	Isopropyl-thio-β-D-galactopyranoside
Kan	Kanamycin
KCl	potassium chloride

KH_2PO_4	potassium dihydrogenphosphate
K_2HPO_4	dipotassium hydrogenphosphate
LB	Luria broth
Lys	Lysine amino acid
MAP kinase	Mitogen Activated Protein kinase
µm	micro molar
NAD+	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NaCl	sodium chloride
NADPH	reduced nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	disodium hydrogen phosphate
NCC27	Nuclear Chloride Channel protein-27 KDa
NEB	New England Biolabs
Ni^{2+} ions	nickel ions
NTA	nitrilotriacetic acid matrix
N-domain	Amino terminal domain
NMR	Nuclear Magnetic Resonance
O.D.	optical density
OH•	hydroxyl radical
ORF	open reading frame
P64	bovine chloride channel protein
PFT	Pore Forming Toxin
PBS buffer	Phosphate-buffered saline
PICOT	protein kinase C interacting cousin of thioredoxin
Phe	Phenylalanine amino acid
PTMD	Putative Transmembrane Domain
Pro	Proline amino acid
PLB	Planner Lipid Bilayer
PVDF	polyvinylidene fluoride membrane
PTM	post-translational modification
RT	room temperature

RT-PCR	Real-time quantitative Polymerase Chain Reaction
RNR	ribonucleotide reductase
RyR	Ryanodine receptor
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate -Polyacrylamide
Ser	Serine amino acid
SEC	Size Exclusion Chromatography
TBE Buffer	tric,boric acis, ethylene diamine tetra acetic acid buffer
TCEP	tris-2-carboxyethyl-phosphine
TEMED	NNNN'-tetramethylethylenediamine
TGM2	transglutaminase-2
Trp 35	Tryptophan 35 in CLIC1
TMD	Transmembrane Domain
Trx	Thioredoxin
TrxR	thioredoxin reductase
UV	Ultraviolet
Val	Valine amino acid
WCLs	Whole Cell Lysates
X	any amino acid

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Abstract

The chloride intracellular ion channel (CLICs) proteins are atypical anion selective channel proteins, as they are principally soluble proteins, with some members now known to also demonstrate enzymatic activity. Structural studies demonstrate that the CLIC proteins share strong structural homology with members of the glutathione-S-transferase superfamily of enzymes, in particular the omega glutathione-S-transferase (GST-Ω) members.

The discovery that the CLIC proteins have the functional ability to act as glutathione dependent oxidoreductase enzymes, also suggested a role for them as cell protective proteins and antioxidants. Therefore, this PhD project aimed to further define the functional activity of the CLIC proteins, in particular CLIC3 as one of more recently identified members.

The principal findings of this PhD project include demonstrating for the first time that CLIC3 has glutathione dependant oxidoreductase activity via its dithiol active enzymatic site. In this study, we directly contributed to the finding that the extracellular activity of transglutaminase-2 (TGM2) is regulated by CLIC3's oxidoreductase activity, and their interaction was dependent upon the redox environment. Furthermore, our *in vitro* studies of CLIC3, were key in helping demonstrate a critical role for secreted CLIC3 in cancer metastasis and tumour cell invasiveness.

To further investigate this oxidoreductase activity of the CLIC proteins we used a bacterial cell model to probe their ability to protect cells against oxidative assault. Recombinant CLIC proteins were expressed in bacterial *E. coli* cells, followed by their exposure to the oxidising agent hydrogen peroxide (H2O2). Expression of CLIC1 by the *E. coli* cells was found to provide increased tolerance of up to 5mM H2O2, while CLIC3

afforded some protection, with no difference seen for cells expressing CLIC4. This work for the first time demonstrates CLIC1 protein acting in antioxidant cellular protection.

The final part of this PhD project pursued the study of the CLIC proteins' glutathionylation activity. Given their close resemblance to the GST-omega-1 proteins, we hypothesised that members of the CLIC family would like them, be capable of deglutathionylation activity. Using a synthetic peptide substrate, our *in vitro* studies demonstrate members of the CLIC family have significant deglutathionylation activity supporting a major role for them in the cellular gluathionylation cycle.

In conclusion, our results provided new insights into the functions of the CLIC proteins as soluble enzymes, with functions in cellular antioxidant protective mechanisms. Most importantly, it points to their role as post-translational regulators of target proteins via their deglutathionylation activity.

Chapter 1

Chloride Intracellular Ion Channel (CLIC) Proteins

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Chloride Intracellular Ion Channel (CLIC) Proteins

1. 1 Chloride Ion Channels

All living cells are surrounded by phospholipid bilayer membranes that separate the cell cytoplasm and its intracellular components from the extracellular environment. Eukaryotic cells have additional intracellular membranes that separate their intracellular organelles and create discrete compartments within the cell. These biological cell membranes are composed of amphiphilic phospholipids, which have a hydrophilic phosphate head group and a hydrophobic tail comprised of two fatty acid chains. Due to this chemical structure, phospholipids spontaneously arrange into lipid bilayers when exposed to aqueous environments. Their hydrophilic head groups remain in the outer bilayer exposed to the aqueous environment, while the fatty acid tails pack together to form a hydrophobic core (1, 2) (Figure 1.1).

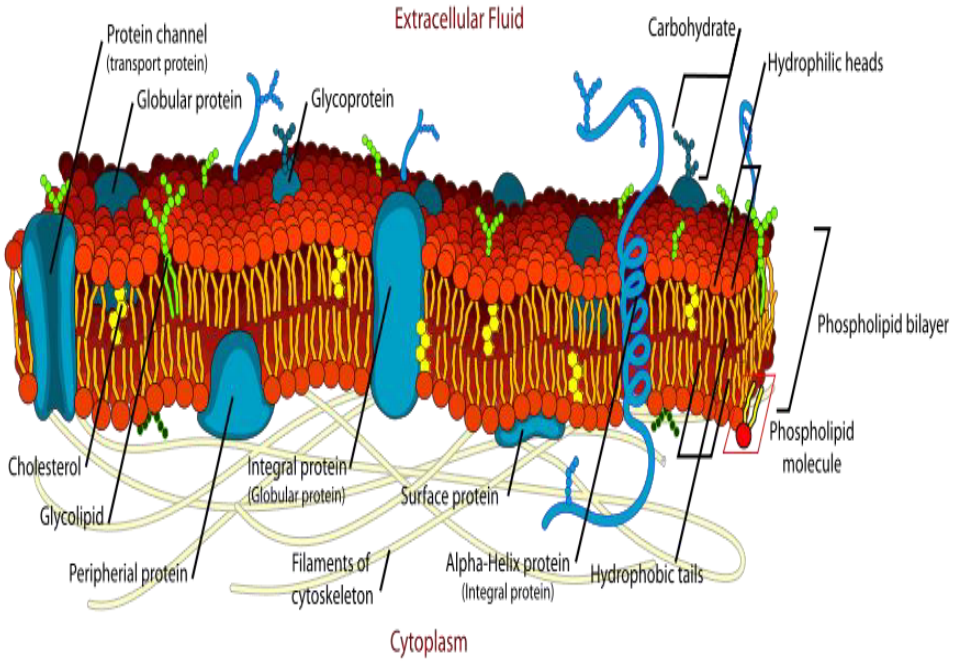


Figure 1. 1 Diagram of a model cell plasma membrane. *Integral membrane proteins (blue) span the phospholipid bilayer (red). The hydrophilic and the hydrophobic regions of the phospholipid bilayer are arranged as two sheets of the membrane. Taken from (1).*

Chloride ions are the most physiologically abundant and predominantly conducted negatively charged ionic species and are essential for the normal functioning of most cells. (3). Chloride ion channel proteins are found in all cell types ranging from prokaryotes to mammalian cells. They are located within the plasma membrane of cells, as well as on the membranes of intracellular vesicles, where they facilitate the transportation of chloride ions across the lipid bilayer. These membrane transport proteins are also amphiphilic and span the entire lipid bilayer. They have a hydrophilic surface that is in contact with the aqueous solution or with the polar head groups of the membrane phospholipids, and a hydrophobic belt that interacts with the alkyl side chains of the phospholipid fatty acid chains in the hydrophobic core of the membrane (1, 4). It is via this structural arrangement that these integral membrane proteins form channels in the membrane, allowing the passage of ions into and out of the cells and which can open and close or "gated" in response to certain stimuli or cellular conditions (5).

Ion channel gating is classified into three categories: voltage gated channels which open and close due to changes in the membrane potential, ligand gated channels that are activated by the binding of specific molecules to their active sites and a third group of ion channels that are gated via mechanical stimuli, for example cell swelling or other mechanical membrane perturbations (6). Chloride channels participate in and regulate different cellular processes including pH regulation, trans-epithelial transport, and regulation of cellular electrical excitability. Genetic abnormalities in the well characterised chloride channel family, the CLC's, results in a number of severe inherited diseases such as myotonia, osteopetrosis, epilesy and deafness (7, 8) demonstrating the

critical role these proteins play in normal cell and whole body physiology. The major classes of chloride ion channels include: the gamma-aminobutyric acid (GABA) and glycine receptor ligand-gated ion channels (9); the chloride ion channels (CIC) family of proteins (9-11) ; the cystic fibrosis trans-membrane conductance regulators (CFTR) (12) and the most recently discovered chloride intracellular ion channels, the CLIC proteins (9, 13, 14).

In mammals, there are nine CLCs. They are localised either in the plasma membrane or on intracellular membranes and can have an ubiquitous or a more restricted tissue distribution (15, 16). The mammalian CLCs 1-7, CLC-Ka, and CLC-Kb, have important physiological roles such as controlling cell excitability (17) transporting chloride ions through epithelial tissue (18) trafficking of receptors necessary for endocytosis (19) and acidification of intracellular vesicles (20, 21). Additionally, mutations in human CLC encoding genes give rise to muscle (22) kidney (21) and bone disorders (23).

The protein CLC-1 is expressed in skeletal muscles and regulates excitability. Loss of function or mutation within CLC-1 causes muscle myotonia (24, 25). CLC-2 is broadly expressed and plays several roles, including cell volume regulation (26, 27), neural cell excitability (28), and transepithelial Cl^- transport (29). CLC-3 is widely expressed and has been suggested to encode (30) or regulate cell swelling (31). CLC-Ka and -Kb are important for transepithelial transport in renal tubules, and mutations in their genes are linked to renal diseases (17, 32). Other CLCs, (CLC-4 to -7) are mainly present in the membrane of intracellular vesicles, including endosomes, lysosomes and synaptic vesicles (5, 33, 34).

There are also other types of chloride channels including the Ca^{2+} activated chloride channels (CaCCs) which were first described in *Xenopus* oocytes in the early 1980s involved in fertilisation (35). Subsequently, CaCCs were found in many other cell types

including neurons, epithelia, muscles, olfactory and photoreceptor cells (5). The most recently discovered group of chloride ion channel are the intracellular chloride ion channel family (CLIC) which are discussed in more detail below.

1. 2 Chloride Intracellular Ion Channel (CLIC) Family

The chloride intracellular channel proteins (CLICs) are atypical anion selective channel proteins. Unlike classical channels (5), CLIC proteins are found in most tissues and cells on their intracellular membranes rather than on the plasma membrane (35-37). CLIC proteins are predominantly localised in the membranes of various intracellular organelles including the nucleus (38) mitochondria (39) secretary vesicles (40) Golgi vesicles (41) and endoplasmic reticulum (ER) (35).

Six CLIC members exist in humans: CLIC1, CLIC2, CLIC3, CLIC4, CLIC5 , CLIC6 (42) (Figure 1.2). All members contain an approximately 240 amino acid residue cassette (43, 44). While, CLIC5 and CLIC6 are two larger variants, with an extended N (amino) terminal domain (45).

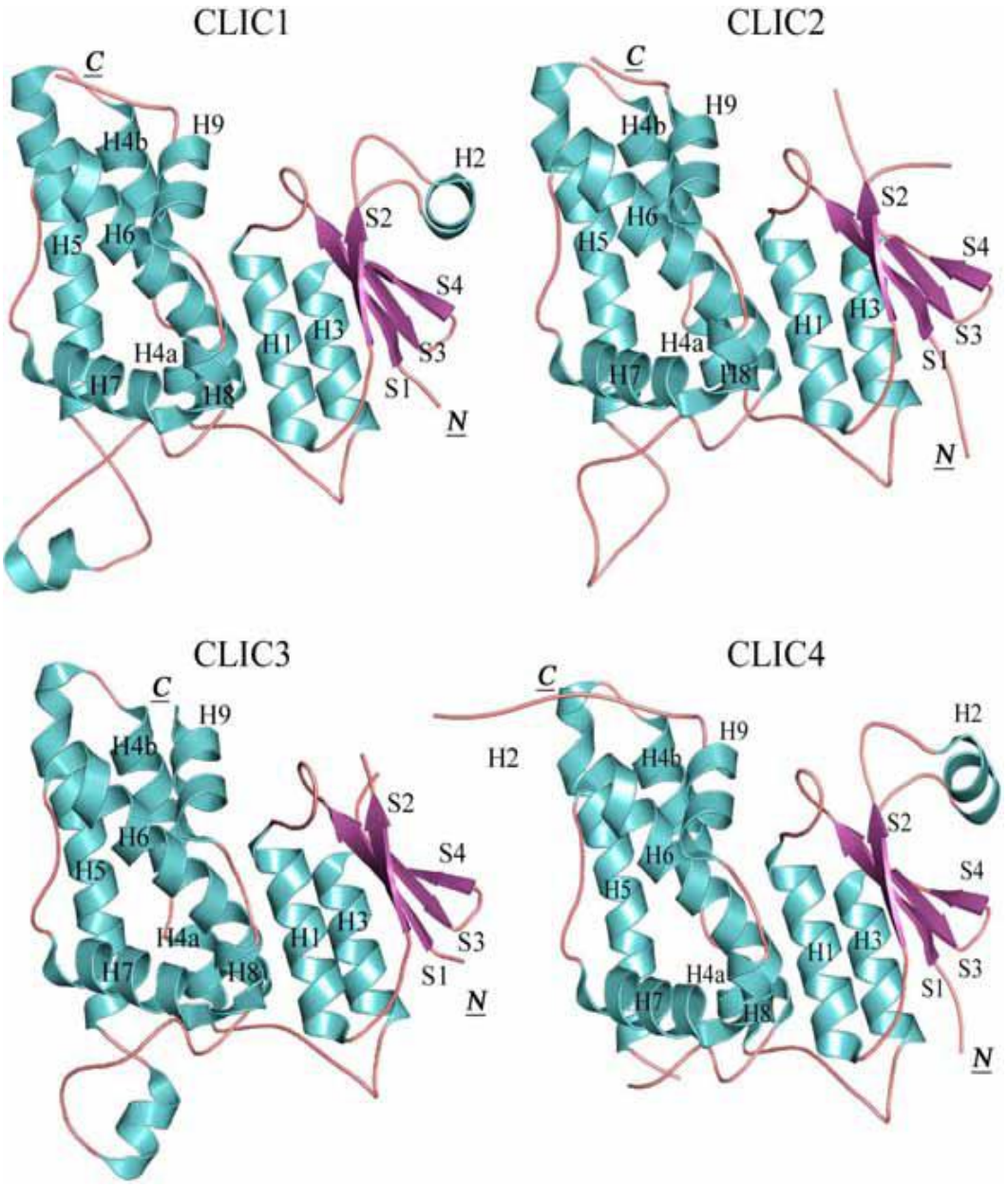


Figure 1. 2 Structures of human CLIC family members determined by X-ray crystallography. *Secondary structural elements are colored and labelled according to type: alpha-helix (cyan, H1-H9), beta-strand (purple, S1-S4) and random coil (pink). The N-terminus and C-terminus are also labeled. Structures shown include CLIC1 (PDB: 1K0M), CLIC2 (PDB: 2R4V), CLIC3 (PDB: 3FY7) and CLIC4 (PDB: 2AHE). Taken from (46).*

CLIC and CLIC-like proteins have been found in other animal species and in plants. Members of the CLIC family have also been identified in many vertebrates such as amphibians, birds, fish and mammals. Six members of the CLIC family have now been shown in vertebrates (CLIC 1-6). Members of the CLIC family display high sequence similarity between them, ranging from 47% to 76% (43, 47) (Figure 1.3). There are also known to be CLIC-like proteins in invertebrates, such as sea squirts, nematodes and insects (1). The proteins EXL-1 and EXC-4 found in the nematode *Caenorhabditis elegans* (*C. elegans*) share a sequence homology of about 43% when compared with human CLIC1, while *Drosophila melanogaster* contains a single CLIC1-like protein (*Dm*CLIC) that shares a sequence homology of about 25% with human CLIC1 (43). Also a single CLIC protein was found in the chordate *Ciona intestinalis* which has 45% homology with the human CLICs (43).

Invertebrate CLIC proteins have lower sequence similarity to the human CLIC proteins (48) and lack some of the conserved N-domain residues; they are also lacking the glutathione (GSH) binding site (located at Cys24 in CLIC1) observed in the human CLIC proteins (49). Instead, they have a divalent metal ion binding site (46, 50). In addition, the invertebrate CLICs contain an extension in their C- domain (46). CLIC-like proteins have now also been found in various plants such as *Arabidopsis thaliana* with a sequence similarity of around 26% to human CLIC1 (51).

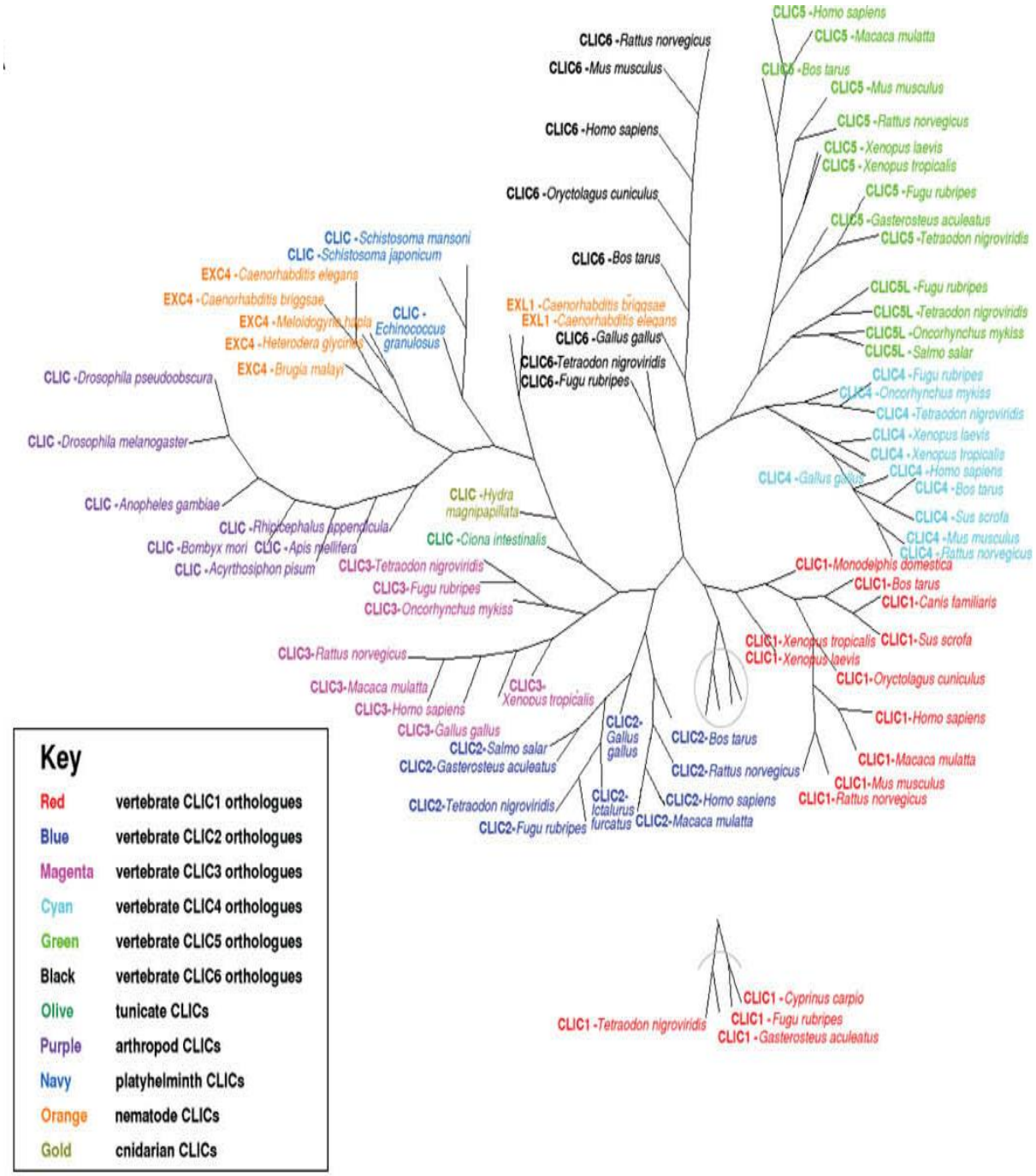


Figure 1. 3 Phylogenetic analysis of CLIC proteins. *The phylogenetic tree of vertebrate CLIC and invertebrate CLIC-like proteins, branches are colored according to the key on the left. Adapted from (43).*

Table 1. 1 summarizes the molecular mass; tissue expression and localisation; cellular roles and molecular functions of human CLIC proteins.

Protein	Sub cellular localisation and tissue distribution	AA length	Molecular mass (kDa)	Cellular roles and molecular function	References
CLIC1 (NCC27)	-Expressed in most body tissues. -Plasma and nuclear membrane. -Intracellular vesicles. - Cytoplasm and nucleoplasm.	241	26.9	-Ion channel activity. -Cell division and apoptosis. -Cell cycle regulation. -promotes cell motility and invasion. -Biomarker for colorectal cancer. - A putative therapeutic target in Alzheimer's disease and cancer therapy. -Associates with AKAP350, PPIɣ2, Sedlin, LSM1 -Functioning as an enzyme.	32,36,38,40,59,65,71-75
CLIC2	-Expressed in most body tissues, except brain. -Plasma membrane and cytoplasm. -Highly expressed in the spleen and the lungs.	243	28.2	-Ion channel activity. -involved in the regulation of cardiac ion channels by modulating the activity of RyR. -Diseases linked to Xq28, Mental retardation.	44,71,76
CLIC3	-Predominantly localise to nucleus and cytoplasm. -Highly expressed in lungs, heart and placenta. -Low expression in kidney, skeletal muscle and pancreas.	207	26.7	-Chloride channel activity. -Regulation of cell growth control. -Protein binding. -Regulation of cell proliferation. - linked to ERK7, a mitogen activated protein kinase (MAP-kinase). -The absence of CLIC3 functioning as a chloride channel makes cells more prone to Listeria infections. -Expressed in bladder cancer and a peptide (CLT1) is able to kill bladder cancer cells through integrin α5β1 integrin and CLIC3. - Acts as an enzyme.	71,77,115,133
CLIC4 (p64H1)	-Plasma, intracellular membranes, endoplasmic reticulum, mitochondria and the cytoplasm of keratinocytes. -Neurons and apical region of proximal tubule cells. -Highly expressed in brain, lungs, liver and skin.	253	28.6	-Chloride channel activity. -involved in angiogenesis and acidification of cells. -induces apoptosis in several cells including human keratinocytes. -Cell differentiation and cytoskeleton organisation. -Associates with tubulin, dynein intermediate chain (DIC), dynamine 1, and actin, AKAP350, PPIɣ2, and Importin-α, Histamine (H3R). -Appears to act as enzyme.	38,52,62,69,76

CLIC5A (P62)	-Microvillus cytoskeleton. -Cortical actin cytoskeleton. -Golgi apparatus.	251	32	-Chloride channel activity. -involved in formation of stereo cilia. - Ion absorption and secretion. -Development of the organ of corti. -Associates with actin, erzin, gelsolin, IQGAP1, PHR and PPIγ2.	41,70,72,78,79
CLIC5B (P64)	-Golgi apparatus. -Plasma membrane. -Cytosol of Hela cells. -Post-acrosomal region of sperm head. - Placenta.	410	46.5		
CLIC6	-Associated with C-terminus of Dopamine D2- like receptor. -Cytoplasm and plasma membrane. -Expressed in the brain, liver, placenta and the pancreas.	704	73	-Chloride channel activity. -Involved in the regulation of body fluid via chloride ions transport. -Dopamine receptor binding. -Involved in the secretion of hormones from cells in pituitary gland. -Associates with D2/D3/D4 dopamine receptor and PHR.	71,80

1. 3 Structural Characteristics of the CLIC Proteins

1.3. 1 The N- and C-terminal Domains of the Protein CLIC1

CLIC1 consists of 241 amino acids with a molecular weight of 26.9 kDa (47). CLIC1 is a monomeric protein and contains ten α-helices and four β-strands, resulting in 47.7% α-helices and 8.3% β-strands in its secondary structure according to X-ray crystallography studies (49). The X-ray crystallographic structure of the soluble form of CLIC1, determined at a resolution of 1.4Å at pH 5.0, was determined to have dimensions of approximately 5.5 nm, 5.2 nm, 23 nm (49). As seen in (Figure 1.4). CLIC1 is comprised of two domains: an all α-helical C-domain and a thioredoxin N-domain. The N-domain includes all four strands of mixed β-sheets (strands β1, β2, β3 and β4) and three α-helices (α1, α2, α3). The C-domain contains α-helices α (4-10) as well as an anionic “foot loop” that is a characteristic feature of the CLIC proteins.

For CLIC1, the active site is located in the N-terminal- domain and contains a central active cysteine residue (Cys24 in CLIC1) (47, 49). It was demonstrated that the presence

of this Cysteine residue is important for the ion channel activity by most of the human CLIC proteins (47).

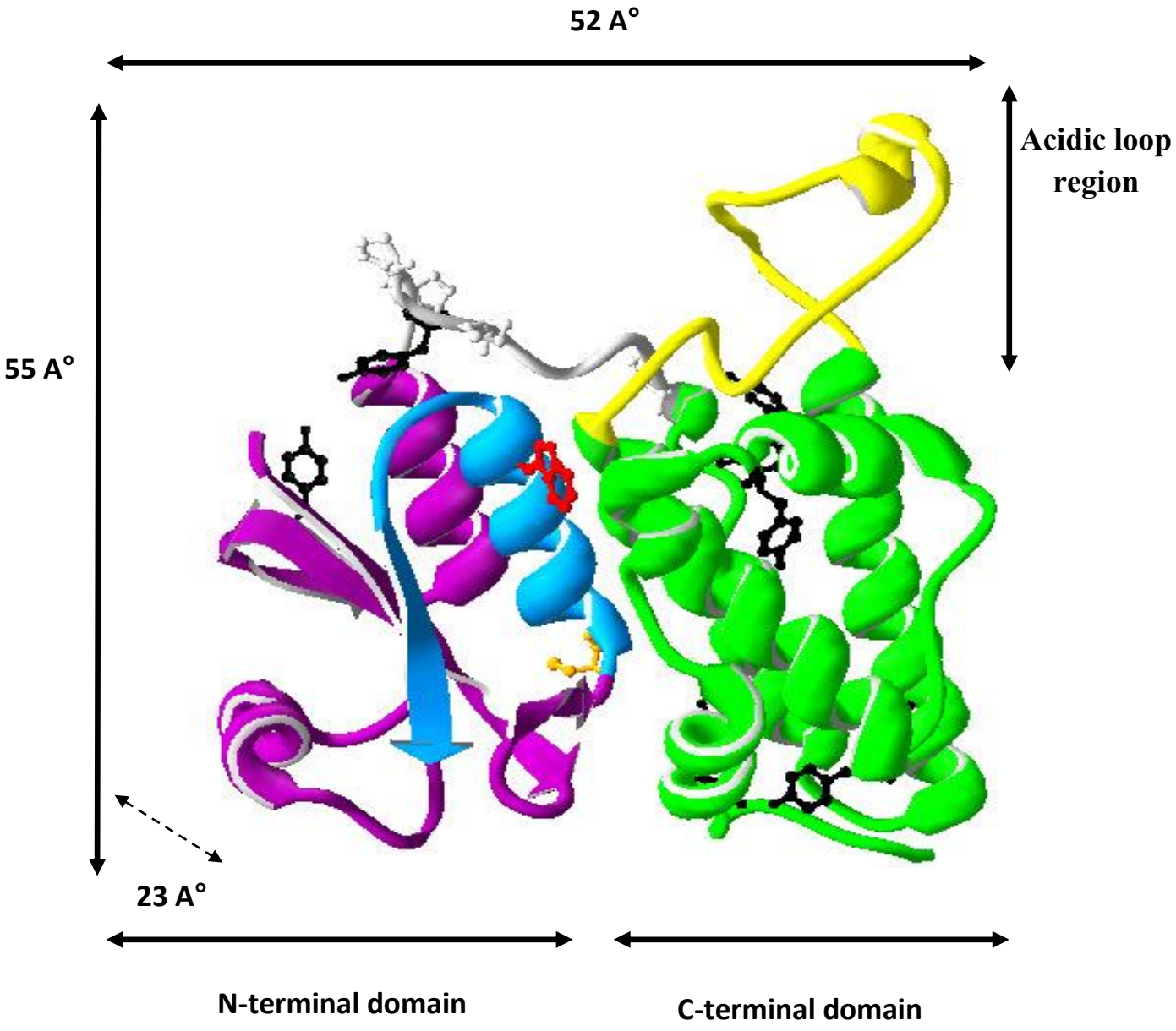


Figure 1. 4 Crystal structure of CLIC1. Crystal structure of reduced CLIC1. *The N-terminal domain containing the thioredoxin fold is indicated in purple and blue and the all α-helical C-terminal domain is indicated in green and yellow. The proline-rich loop connecting the two domains are indicated in grey. The active site Cys24 is indicated in orange. The single tryptophan residue, Trp35 is in red and the eight tyrosines (two in the*

N-terminal domain and six in the C-terminal domain) are in black. The negatively charged loop is shown in yellow and the helix that is considered likely to traverse the membrane is indicated in blue. This was modified from (49, 52).

CLIC1 contains a single tryptophan, Trp35, at the putative N-terminal transmembrane domain. Furthermore, eight tyrosine and six cysteine residues are distributed throughout the CLIC1 protein molecule. There is also a proline-rich loop (Cys89-Asn100) connecting the N- and C- domains. This proline-rich loop is the origin of the domain interface plasticity in the protein's structure (49). It was proposed that the N-domain would need to move away from the C-domain, to initiate membrane insertion of CLIC1. As such, the loop which leads to flexibility between the two domains is likely to be involved in the transition between the soluble and the transmembrane forms of CLIC1.

1.3. 2 The Putative Transmembrane Domain of CLIC1

CLIC proteins contain two hydrophobic regions: first is the putative transmembrane domain and the second is the proline rich interdomain loop (53). The N-domain of CLIC1 has the putative transmembrane domain (PTMD) located between Cys24 - Val46, and spanning α-helix 1 and β-strand 2 (50) (Figure 1.5). The PTMD arises from the hydrophobicity and the α-helical propensity in the N-domain (53, 54). The PTMD for CLIC1 was found to form a network of contacts between the α1, α3 and α5 helices via the residues Glu81-Arg29 and Glu85-Lys37 (55).

Human	24	46
CLIC1	CPSFQRLFMVLWLKGVTFNVTTV	
CLIC2	CPFCQRLFMILWLKGVKFNVTTV	
CLIC3	CPSCQRLFMVLLLKGVPFTLTTV	
CLIC4	CPFSQRLFMILWLKGVVFSVTTV	
CLIC5	CPFSQRLFMILWLKGVVFNVTTV	
CLIC6	CPFSQRLFMILWLKGVIFNVTTV	

Figure 1. 5 Structure-based sequence alignment of the putative transmembrane domain (PTMD) of the CLIC proteins. *The PTMD at the N-terminal domain is conserved amongst all the human CLIC proteins. In CLIC1 the PTMD starts from Cys24 to Val46. (49).*

The PTMD joins α1 and α3 helices of the N-domain with the α5 helix in the C-domain, by spanning the two domain interfaces. In the PTMD of CLIC1, there are two conserved phenylalanines (Phe26 and Phe41), with ten non-polar residues (Leu30 to Val39) located between these two conserved phenylalanine residues. The remaining six to seven are polar residues. Furthermore, the presence of the unique Trp35 in the amphipathic α1 helix is the main contact element forming the putative transmembrane domain and making the most contacts between domain 1 and domain 2 (56).

There is a proline rich interdomain loop in CLIC1 protein has been located at the C-terminal domain and mostly comprising α- helix 6 and part of the loop preceding it (53). The presence of these two hydrophobic regions in CLIC1 protein are the connection between the N- terminal and C-terminal domains (49). It has been demonstrated that the modulation in the proline- rich loop and the relative orientation of the two domains are inter-connected and supply abundant flexibility, which can potentially act to simplify the

structural rearrangement of the soluble monomeric structure into an integral membrane form.

The PTMD has a crucial role in the biophysical properties of the ion channels function of the CLIC proteins. Mutations in these regions can affect different features of ion channels like their conductance, gating or ion selectivity (57, 58). Mutations in the transmembrane domain caused by replacing the two charged residues (Arg 29 Ala and Lys 37 Ala) where created in CLIC1. These mutations were evaluated by using different methods including: artificial lipid membranes and cell – attached and whole – cell electrophysiological recordings of transiently transfected HEK cells (55). The results showed that changing the only charged residues in the putative transmembrane domain of CLIC1 with neutral alanine, lead to alteration in its electrophysiological ion channel properties (55).

1. 4 CLICs as membrane proteins

Several proteins have now been found to exist in both soluble and membrane-bound forms. These proteins have a property that distinguishes them from typical membrane proteins, as they are soluble proteins without a leader sequence for membrane targeting but are able to insert into or associate with membranes. These proteins include some of the bacterial toxins (59), porins (60), the eukaryotic proteins Bcl-2 (61-63), Bax (64) and the CLIC family (65).

CLIC1 exists both in soluble form and as an integral membrane protein, however it also possess the unusual ability, to shift between two stable tertiary protein conformations, an ability described as “metamorphic” (66). These two soluble forms have been resolved by X-ray crystallography, as the reduced monomeric form (67) and in its oxidized state (67). However, much less is known about the structure of the integral membrane form. Also, the mechanism of CLIC1 membrane-insertion remains unknown.

The crystal structure of soluble CLIC1 was solved in reducing and oxidising environments by Littler and colleagues (47). This indicated that under reducing conditions CLIC1 adopts a monomeric GST–like structure, containing a glutaredoxin-like active site. However, in an oxidative environment, CLIC1 adopts a non-covalent dimeric state. The dimer form arises via the formation of an intramolecular disulphide bond between cysteine 24 and cysteine 59 within each monomer. The assumption of the dimeric state entails dramatic structural changes, where most of the N-terminal domain adopts a different secondary and tertiary structure. It was shown that CLIC1 undergoes a conformational change to insert into artificial bilayers. A study using fluorescence resonance energy transfer (FRET) spectroscopy to measure the distances between the tryptophan in position 35 located in the N-terminal domain (suggested to be transmembrane domain) and the three conserved cysteines (Cysteine 89, Cysteine 178 and cysteine 223) located in the C-terminus in the presence of membranes or in aqueous solution. This indicated that in the presence of a lipid bilayer conformational unfolding occurs between the N- and C-terminus, which was not observed in aqueous solution (68). The insertion of CLIC1 into the membrane has been shown to be regulated by redox conditions (43, 47, 67, 69). The putative trans-membrane domain is very important in membrane insertion of CLIC1, especially the putative trans-membrane domain which is formed by residues Cys24 – Val46 (49). Furthermore, a study using CLIC- like protein showed that the first 55 residues at the N-terminal are very important for membrane insertion with the tryptophan (Trp/W35) residue localised within the membrane (50) (Figure 1.6).

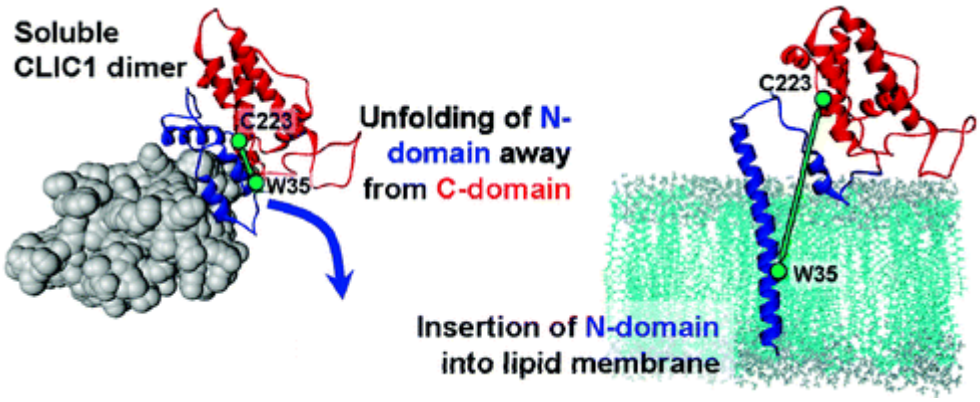


Figure 1. 6 Membrane insertion model of CLIC1 protein. *The N-terminal domain of CLIC1 inserts into the lipid bilayer (highlighted in blue), while the C- terminal domain remains cytoplasmic (highlighted in red). Taken from (70).*

Oxidizing conditions have been shown to enhance CLIC1 channel activity, as well as its insertion into artificial membranes. The N-domain of CLIC1 contains a glutathione (GSH) binding site, with an active Cysteine located within this site, (Cys24). which is located at the beginning of helix α2 in the N-domain (49). Furthermore, it has been suggested that CLIC1 may use its GSH-binding site to recognise the mixed disulfide bond of some GSH-modified proteins and thus target the chloride channel to a specific subcellular location (49). This however is not supported by mutation studies that show the CLIC1 C24A mutant is still able to insert into membranes, even though the critical Cys24, assumed as the redox control of insertion was removed (69).

Under oxidizing conditions, CLIC1 has been shown to form a reversible non-covalent dimer with an intramolecular disulfide bond between Cys24 and Cys59 (Figure 1.7 A) (47). The dimer structure is reversible, demonstrated by conversion of dimeric CLIC1 protein back to its monomeric state when exposed to 5 mM of the reducing agent DDT (47).

The dimer seems to preferentially be formed only the absence of lipids, while in the presence of a lipid membrane, the newly exposed hydrophobic surface of CLIC1 monomers under oxidizing conditions prefer to interact with the membrane (47). The dimeric CLIC1 has been demonstrated to undergo a dramatic structural rearrangement from its monomeric state. Instead of the four β-strands, the N-terminal domain becomes all α-helical and loops to form a hydrophobic interface. Two soluble CLIC1 monomers can thus interact via their hydrophobic interface, to form a CLIC1dimer (Figure 1.7 B). From this, it has been suggested that CLIC1 adopts a structure similar to this dimer structure, exposing it hydrophobic interface to then insert into the membrane (43). This is supported by evidence showing that both monomeric and dimeric CLIC1 have the ability to insert into the membrane and form functional ion channels in artificial membranes (47).

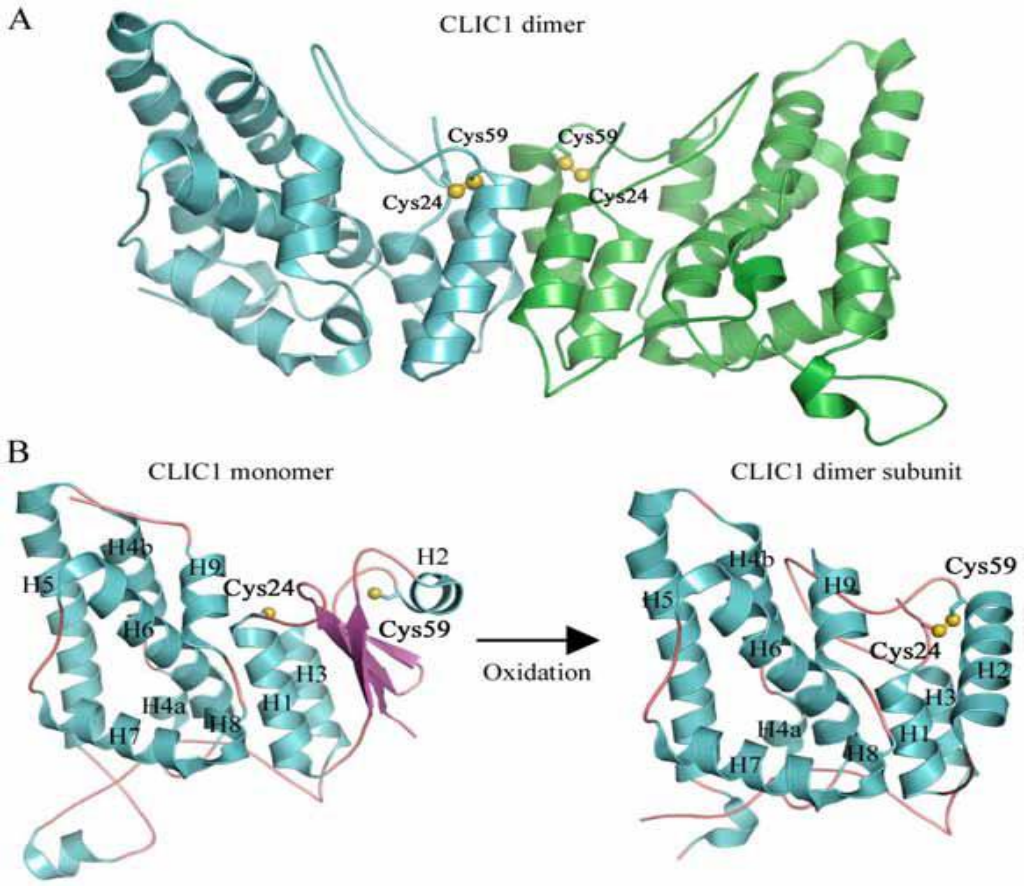


Figure 1. 7 The oxidized CLIC1 dimer.

A) The all alpha-helical CLIC1 dimer showing the location of critical cysteine residues (Cys24 andCys59) that form the intramolecular bonds that is unique to CLIC1. The two subunits are shown in cyan and green.

B) The CLIC1 monomer is shown coloured by secondary structure elements, with the location of Cys24 and Cys59 labelled. CLIC1 undergoes a redox controlled structural rearrangement which facilitates the formation of a disulphide bond between Cys24 and Cys59, which is shown in the CLIC1dimer subunit.

Interestingly, the oxidation of monomer enhances the insertion of CLIC1 protein into the membrane compared with the oxidation of dimer (67). It was concluded from this, that the dimerization process is likely to not be essential for membrane insertion (67).

Furthermore, the formation of dimer by the strong interaction between the hydrophobic interfaces may even cause steric hindrance for subsequent CLIC1 membrane insertion (67). Further evidence suggesting that redox state is not the central regulatory mechanism for membrane insertion comes from the fact that the Cys59 residue involved in stabilising the CLIC1 dimer structure, is not conserved in any of the other five human CLIC proteins. It is thus, difficult to confirm the role of the intramolecular disulfide bond in membrane insertion of the CLIC family.

Additionally, EXC-4 - the CLIC-like protein found in *C. elegans* - can localise to luminal intracellular apical membranes and a 66 amino acid residue of the N-terminal domain is required for this. This N-terminal helical portion of the protein has therefore been termed a putative transmembrane helix (48, 50). Furthermore, studies of CLIC1 suggest that CLIC proteins might only insert into lipid bilayers under oxidising conditions. However, Singh and colleagues have found that CLIC4 inserts into membranes under both oxidising and reducing conditions (71). Consistent with this, CLIC2 inserts into membranes at lower pH and in a way that is insensitive to redox state (72). Therefore, further studies need to be conducted to identify under which conditions specific CLIC family members insert into the lipid bilayers and whether the insertion into the membrane has any physiological role *in vivo*.

1.4. 1 The Role of Sterols on CLIC1 membrane insertion

Most eukaryotic cells contain and synthesize sterols. Cholesterol is the unique sterol of vertebrates, while ergosterol is the major sterol of most fungi, some unicellular algae and higher plants (73) (Figure 1.8).

Sterols are membrane constituents and play an important role in lipid biosynthesis pathways. It is well-known that sterols play a crucial role in condensing the membrane bilayer by regulating membrane fluidity and permeability (73, 74).

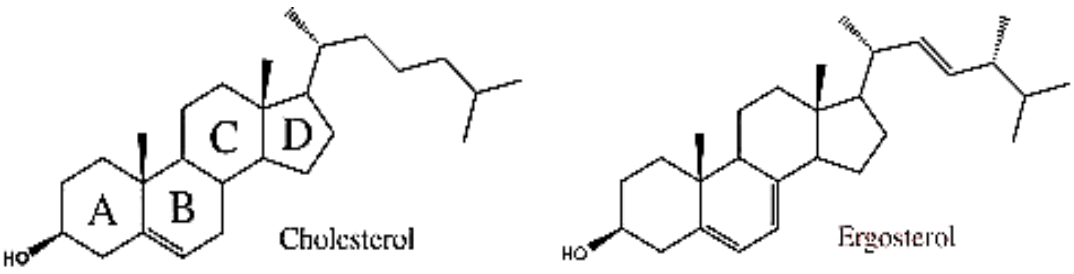


Figure 1. 8 Structure of cholesterol and ergosterol.

There are known amiphitropic proteins, such as, the cholesterol-dependent cytolysins (CDCs) which have the distinct ability to post-translationally insert spontaneously into lipid membranes (38). These proteins are a type of pore forming toxins (PFT) that include proteins such as listeriolysin, streptolysin, perfringolysin and pneumolysin, and are generally secreted by species of gram-positive bacteria (75). As previously described, the CLIC proteins are also considered an example of spontaneously inserting membrane proteins.

A recent study by our group at UTS investigated the ability of the membrane auto-inserting proteins, such as α-hemolysin, listeriolysin-O and CLIC1, to form conductive channels in a tethered lipid bilayer system. The functional channel activity of these proteins was determined by using impedance spectroscopy, where the changes in the lipid bilayer conductance demonstrate the proteins functional activity upon insertion (76). The results showed that the different concentrations of cholesterol in the membrane affect the

ability of CLIC1 to form conductive channels in the tethered bilayer membrane. In addition, Langmuir monolayer file experiments confirmed the importance of cholesterol for CLIC1 to auto-insert into the membrane (76).

The motif GXXXG (at position, G18AKIG22) is present in the amino acid sequence of CLIC1, adjacent to its putative transmembrane domain and found to be highly conserved among the members of the human CLIC family. It has been speculated that this motif may be involved in CLICs interaction with the cholesterol and may play a crucial role in the assembly of multimeric structure of CLIC1 in membranes. In addition, the presence of cholesterol facilitates the transition of soluble CLIC1 into membranes to form an ion channel conductive state (76).

The motif (GXXXG) is also present in members of the CDCs such as listeriolysin 529aa protein (G91YKDG95) and perfringolysin-O 499aa protein contains two copies of the motif (G68KKAG72 and G151KVSG155) and intermedilysin 532aa protein (G163LKNG167). This same motif is also present in the α-hemolysin 240aa protein (G84ASTG88). In addition, the amino acid lysine (K) is present in the CLIC1 and all of the CDC family members. This indicates that the motif GXXXG, likely has a crucial role in the membrane interactions of these proteins and their associations with the cholesterol (76).

1. 5 Structural Similarities between the GST Superfamily and CLIC Protein Family

Most recently, the CLIC family has been classified as belonging to the glutathione-S-transferase (GST) protein super-family. The GST super family is a group of soluble homodimeric proteins with a thioredoxin fold in their N-domain and an all-helical C-domain. Based on structural predictions using its primary sequence by Dulhunty et al., (45), CLIC1 was proposed to be a member of the GST super family. This was then

confirmed by Harrop *et al,* 2001 (49) who determined the crystal structure of CLIC1, showing the characteristic structural fold of the GSTs was also adopted by the CLIC proteins.

The GSTs are grouped into classes including alpha, beta, delta, epsilon, theta, zeta, mu, pi, tau and omega (Figure 1.9). Members of this super family are mostly found to exist as dimeric proteins in the cellular cytosol, with a molecular weight of around 24-25 kDa for each subunit (77, 78).

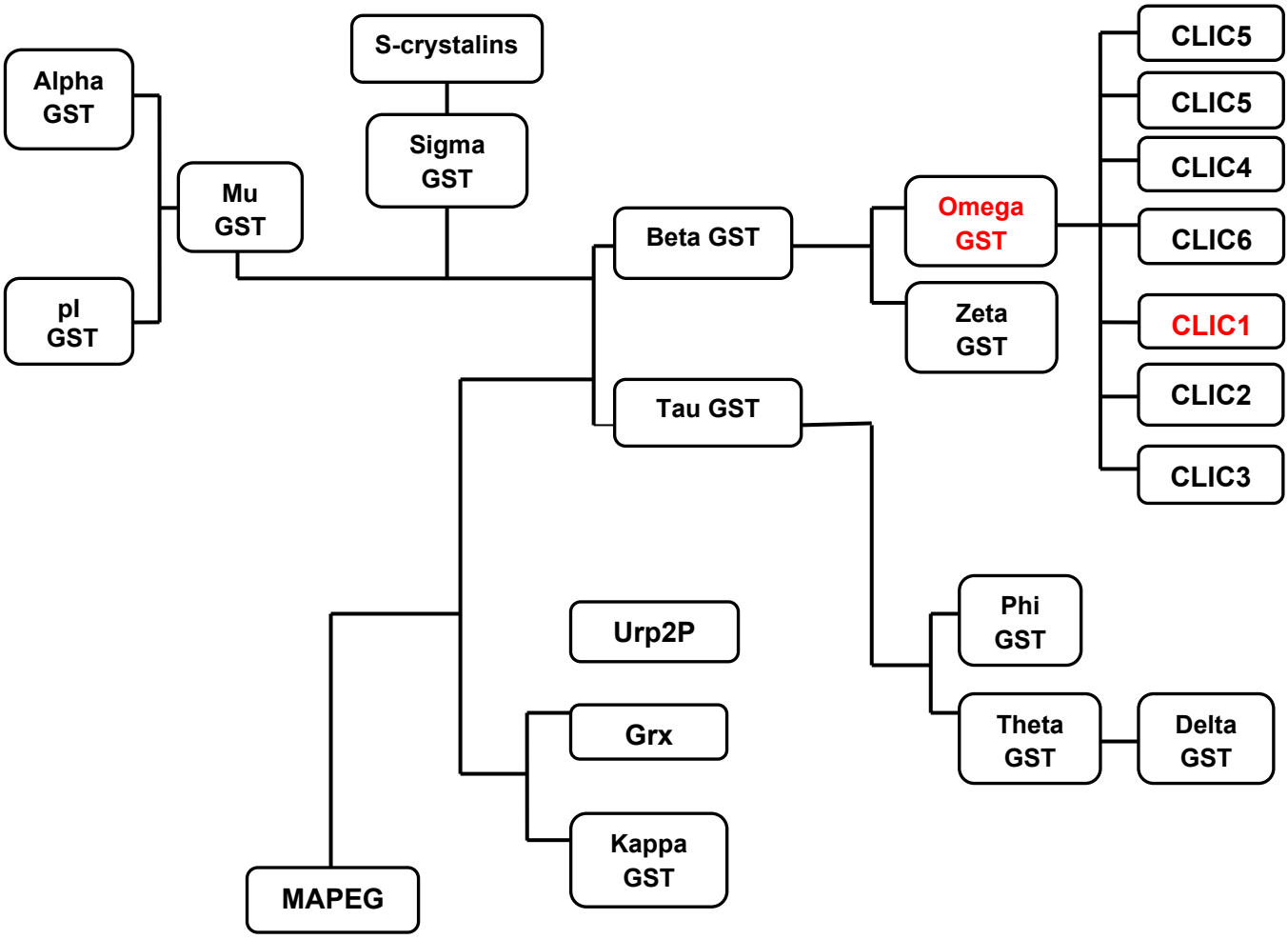
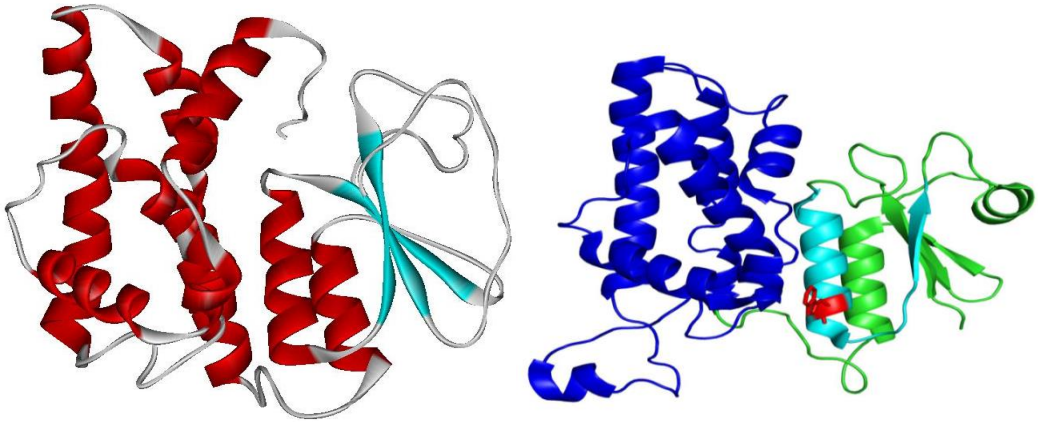


Figure 1. 9 Dendrogram of the GST super family. *Sequence relatedness between the members of the CLIC family adapted from (79) And the members of the GST family adapted from (80). This was modified from (79, 80).*

CLIC1 most closely resembles the human Omega-GST-1 (GST Ω1-1); they have similar number of amino acids, approximately 241 (Figure 1.10) (47, 49). Furthermore these two proteins contain a single cysteine residue (Cys24 in CLIC1 and Cys32 in GST Ω1-1) in their active sites and have the ability to form a mixed disulphide bond with glutathione (47, 49, 81-83). In addition, the inhibitor indanyloxyacetic acid-94 (IAA94) which inhibits the function of the CLICs, is also, an inhibitor for the GSTs (84).



A) *GST- Ω 1* **B) *CLIC1***

Figure 1. 10 Comparison of A) GST Ω 1-1 and B) CLIC1 structure. *CLIC1 and GST Ω 1-1 consist of N-terminal domain with the thioredoxin indicated in gray in GST Ω 1-1 and in green in CLIC1. The all α-helical C- terminal is indicated in red in GST Ω 1-1 structure and in blue in CLIC1 (47, 82).*

1. 6 Antioxidant and deglutathionylation enzymes

Glutathione exists in virtually all cells in the millimolar concentration range. It is the major non-protein thiol compound that acts as an inherent antioxidant, and works together with oxidized glutathione (GSSG) as an intracellular redox buffer (85). GSH is a

tripeptide, containing a cysteine, a glutamate and glycine held together through unusual peptide bonds, in which the carboxyl group of the glutamate side-chain is linked to the amine group of cysteine. While, the carboxyl group of cysteine is linked to the amine group of glycine in a conventional manner.

In mammalian cells, the concentration of GSH ranges from 1~5 mM depending on the cell type. The majority exists in the form of GSH with only a small portion existing as GSSG. ROS tend to accept electrons so that GSH acts as an antioxidant by serving as an electron donor. In this process, glutathione is turned into GSSG. Under physiological conditions, the GSH: GSSG ratio is around 50:1 to 100:1 but this ratio can change drastically in pathological conditions e.g., oxidative stress.

Glutathione can also be added/removed from target proteins via a process known as de/glutathionylation which is catalyzed by specific enzymes. Glutaredoxin (Grx) is suggested to be the major deglutathionylating enzyme in mammalian cells (86, 87). The mammalian cytosolic form of Grx (Grx1) is exclusively selective for protein-SSG compared with other forms of disulfides (e.g. S-S disulfide bond, S-nitrosylation, etc.). It is very effective in reducing protein-SSG, thus is considered as a specific deglutathionylating enzyme. Moreover, in Grx1 knockout mice, no deglutathionylating activity was detected (86), further supporting this critical role. Besides Grx1, other enzymes have been implicated in deglutathionylation or serving in antioxidant defence system, including thioredoxin (Trx2), peroxiredoxins (Prx3/5), thioredoxin reductase (Trxr2), glutathione reductase (GR). However, the specificity and the specific substrates of these enzymes are still not well understood (88).

Thioredoxin and Glutaredoxin proteins share a number of structural similarities. Their active site residues are located within their N-terminal domain (89). Based on their own disulfide reducing system, the thioredoxin system (90) and the glutathione system (91)

play a crucial roles in cell metabolism and DNA synthesis (92) and in redox control for cellular function (93).

Both NMR spectroscopy and the X-ray crystallography have been used to investigate the structures of dithiol and monothiol Glutaredoxin. Structurally, the Glutaredoxins belong to the Thioredoxin fold family of proteins. This motif is composed of a four stranded ß-sheet surrounded by three α- helices (Figure 1.11), which is also conserved by the GSTs and the CLICs proteins. The active enzyme site which is located within this fold is, called the G-site (86) and it commonly utilizes the N-terminal cysteine as its active residue. Both Thioredoxin and Glutaredoxin families share similar active site motifs either the dithiol (Cys-X-X-Cys) or monothiol (Cys-X-X-Ser) motif (94).

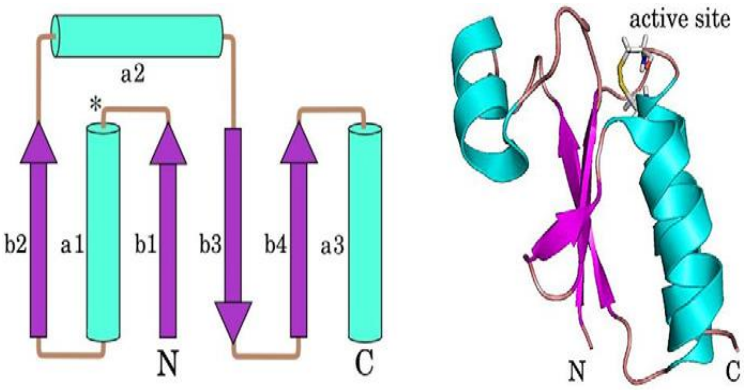


Figure 1. 11 Glutaredoxin structure. *Bacterial glutaredoxins exhibit the most basic representation of the thioredoxin fold (left site). The structure of oxidized E. coli Grx1 is shown. Taken from (94).*

This glutaredoxin- like motif with a redox-active cysteine residue is present in the CLIC proteins. It can form a disulphide bond with GSH, similar to the GSTO Ω1-1 class of proteins (43, 49). This active cysteine is frequently contained within a CxxS or CxxC

motif which is located at the N-terminal domain. Most of the CLIC proteins contain the CPFS motif; however, CLIC2 and CLIC3 are exceptions, as they contain the thioredoxin-like motif with the double cysteine (CPFC). In CLIC2 and CLIC3, these two cysteines are capable of forming an intramolecular disulphide bond (95) (Figure 1.12).

```
Grx-1 --------------------MAQEFVNCKIQPGKVVVFIK--------PTCPYCRRAQEI   32
Grx-2 E----SNTSSSLENLAT---APVNQIQETISDNCVVIFSK--------TSCSYCTMAKKL   86
Grx-3 DIIKELEASEELDTICPKAPKLEERLKVLTNKASVMLFMKGNKQE---AKCGFCKQILEI   270
GST-Ω SA-------RSLG--------KGSAPPGPVPEG--------SIRIYSMRFCPFAERTRLV   41
CLIC1 --------------------------MAEEQPQVELFVKAGSDGAKIGNCPFSQRLFMV   33
CLIC2 --------------------MSGLRPGTQVDPEIELFVKAGSDGESIGNCPFCQRLFMI   39
CLIC3 ---------------------------------KLQLFVKASEDGESVGHCPSCQRLFMV   48
CLIC4 ALS--M---------------PLNGLKEEDKEPLIELFVKAGSDGESIGNCPFSQRLFMI   44
CLIC5 T--------------------DSATANGDDSDPEIELFVKAGIDGESIGNCPFSQRLFMI   41
CLIC6 AAR--VNGRREDG--------EASEPRALGQEHDITLFVKAGYDGESIGNCPFSQRLFMI   478
```

Figure 1. 12 Conserved G-site motif in members of the CLIC family*. Multiple sequence alignment of human proteins: CLIC 1-6, GST-omega and Grx1-3. Highlighted in grey is the glutaredoxin/thioredoxin active site motif (G-site) (Accession numbers: CLIC1 (CAG46868), CLIC2 (CAG03948), CLIC3 (CAG46863.1), CLIC4 (CAG38532), CLIC5 (AAF66928), CLIC6 (NP_444507), GST-omega (AAF73376), Grx-1 (BAAO4769), Grx-2 (AAK83089) and Grx-3 (AAH0528289) obtained from ClustalW.*

Moreover, studying the crystal structure of the CLIC1/GSH complex, revealed that the GSH group is poorly ordered and only makes a few contacts with CLIC1 (low affinity binding) (42), this also led to the observation of the existence of an opening or an "empty slot" located near the GSH binding site of the CLIC proteins (as shown in Figure 1.13) (43, 49, 96). Above all, it was speculated that this empty site could be the binding site for an extended macromolecular chain, perhaps a polypeptide molecule that may function as a substrate.

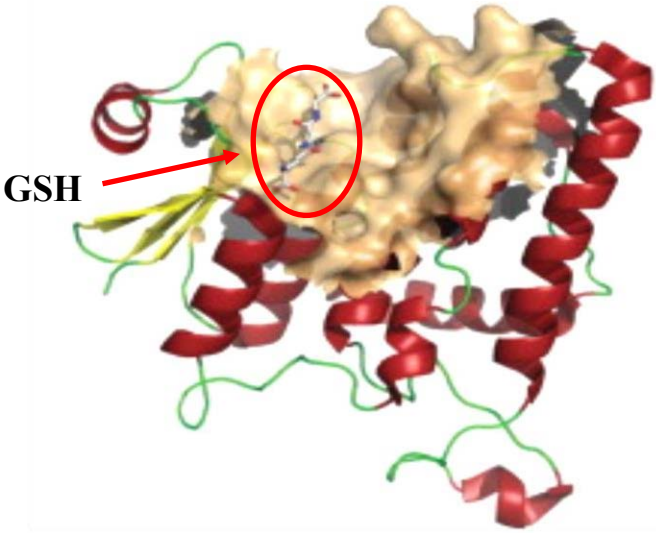


Figure 1. 13 Glutathione (stick model) in CLIC1 protein as shown with red arrow. *In addition to the existence of an opening or an empty site near the GSH binding site, this can be a binding site for macromolecules or polypeptides. Taken from (43).*

Using a range of substrates via *in vitro* assay systems, members of the CLIC family were demonstrated to have oxidoreductase activity, this included the substrate dehydroascorbate (DHA) to ascorbate (97). This demonstrated dehydroascorbate reductase (DHAR) activity, supports earlier homology studies that deemed CLIC1 as closely related to the plant DHAR proteins (98, 99). The plant DHARs adopt a three-dimensional structure similar to the soluble form of CLIC1 protein (51, 98) (Figure 1.14). It has been noted that CLIC-like DHAR protein, PtoDHAR2 from the *Populus tomentose* plant demonstrates oxidoreductase activity and that substitution of the Cys20 residue which is located in its predicted GSH binding site resulted in abolishment of its reductase activity (98). These studies are also supported by our studies for the CLIC1 mutant (C24A), where its activity was diminished when the redox active site Cys24 was substituted with an alanine residue. Further support that these are CLIC-like proteins in

plants, arises from a study showing that cloning of the DHAR from *Arabidopsis thaliana*, AtDHAR1, and transiently expressing the protein in mammalian cells resulted in CLIC-like ion channel activity (51).

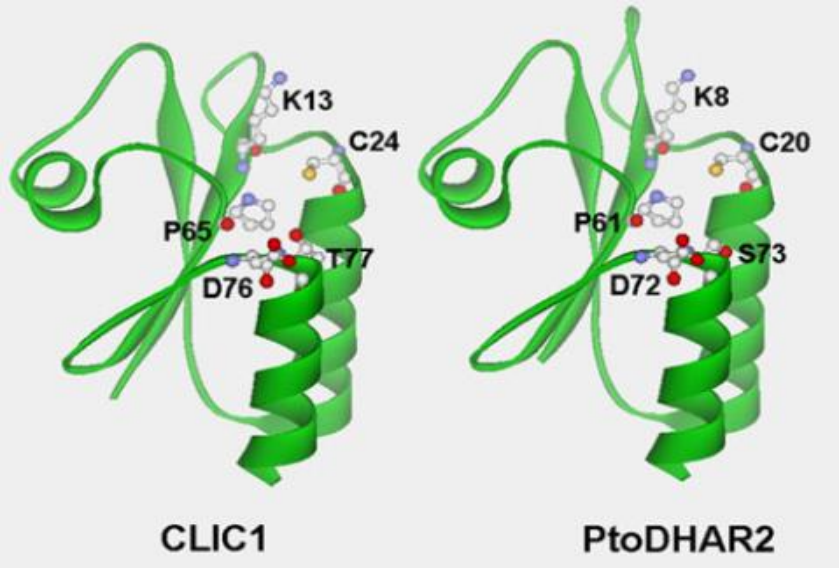


Figure 1. 14 Structural comparison of the N-terminal domain between PtoDHAR2 and the intracellular chloride channel proteins CLIC1. Taken from (98).

1. 7 The Predicted Cellular Protective Effects of the CLIC proteins against Oxidative Stress

Oxidative stress means an imbalance between reactive oxygen species (ROS) and the availability and activity of antioxidants. Generally, oxidative stress is characterized by overproduction of reactive oxygen species (ROS), which include: hydrogen peroxide, superoxide radicals and hydroxyl radicals (100). These excess levels of ROS in turn result in modification of lipids, proteins and DNA, which causes damage to cells and tissues (101). Since oxidative stress is sustained in cell death and necrosis, it also plays an important role in the pathogenesis of neurodegenerative disorders, cancer and

cardiovascular diseases, and may be particularly important in pathologies associated with inflammation (101).

ROS can induce various reversible oxidative modifications to target Cysteine residues of susceptible proteins. Reversible modifications under moderate oxidative stress may protect proteins against permanent damage and modulate their functions (102). Modification of Cysteine residues results in their conversion to sulfonic or sulfenic acids, and formation of intra- and inter- molecular disulfide bonds in proteins, as well as formation of disulfide bonds between protein thiols and low molecular weight thiols such as Cysteine and glutathione (GSH) in a post-translational protein modification process called glutathionylation (102).

The Thioredoxins were the first antioxidants identified in cells, and are known to act as general protein disulfide reductase enzymes (103, 104). Similarly Glutaredoxins have been proposed to play a major role in the response to oxidative stress (105). Glutaredoxins can protect cells against oxidative conditions, with evidence for a protective role of these proteins reported via the use of biochemical (106, 107) and genetic approaches (108, 109).

Members of the Glutaredoxin family and the protein GSTΩ1-1 are able to catalyse protein glutathionylation in order to maintain cellular sulfhydryl homeostasis (110). Glutaredoxin proteins catalyse both the formation and the reduction of mixed disulfides between protein thiols and GSH (111, 112). This indicates that the glutaredoxins also play a crucial role in the reversible glutathionylation of proteins. Given structural studies have shown CLIC1 binds GSH via an glutathione binding site (49) and the recent discovery that the CLICs also have GSH dependent oxidoreductase activity (97, 113) , it has been speculated that glutathionylation is a mechanism by which the CLIC proteins may regulate their ion channel activity and/or other cellular processes (43, 114).

Due to high levels of GSH inside the cell's cytosol, the intracellular environment is in general, highly reduced. Significantly, oxidative stress can alter this environment allowing exposed cellular thiols to become oxidized (115). Thiol oxidation causes a significant production of reactive oxygen species (ROS) (103); increased generation of ROS and superoxides, leads to cellular damage and can induce apoptosis. Therefore, Gluathionylation, which is the formation of mixed disulfides bond between glutathione (GSH) and a cysteine residue of protein, plays a crucial role in cellular homeostasis. It is a post translational modification which controls protein function and also serves as a reversible protective mechanism of protein thiols that can occur under oxidative stress (105, 116), to protect cysteine residues from oxidative damage (110).

In plants, Ascorbate (ascorbic acid or vitamin C, AsA) is the major antioxidant and known as a cofactor in many enzymatic reactions. Ascorbic acid provides a defence mechanism against oxidative stress. Environmental stressors such as drought, salinity, ultraviolet light and extreme temperature induce formation of reactive oxygen species. However, AsA is continuously oxidizing the scavenging reactive oxygen species generated by environmental stressors (117, 118). It has been reported that the overproduction of oxidized ascorbate by dehydroascorbate reductase (OsDHAR) from *Oryza sativa L. japonica* increased the recycling of ascorbate, which catalyses the glutathione (GSH)-dependent reduction of oxidised ascorbate (dehydroascorbate, DHA). Therefore, the CLIC-like DHAR proteins in these plants, act to regenerate a pool of reduced ascorbate and detoxify reactive oxygen species (119).

The work undertaken as part of this PhD project investigated the role of CLICs as cell protective agents. For our studies, we have used a model system to test whether the CLIC proteins can protect bacterial cells from such oxidative stress. Hydrogen peroxide added to the growth media, was used to evoke such oxidative stress in the bacteria. According

to our findings, CLICs were able to protect cells against oxidative damage (unpublished data) and circumvent oxidative stress in a manner similar to Grx-1 (120).

These findings support the hypothesis that soluble CLIC proteins function as antioxidant and as oxidoreductase enzyme in cells. It has been reported that some of the Glutaredoxin systems participate in protection against oxidation or repairing sensitive sulfhydryls for maintaining an adequate redox state of proteins in the intracellular environment and thus for regulating various cellular activities (105, 108, 121). Like the Grxs, CLICs appear to exhibit similar oxidoreductase activity. From these results, one could speculate that members of the CLIC family contribute to maintaining an adequate redox state of proteins in the intracellular environment and via this process also serve to regulate a variety of cellular activities.

In conclusion, the CLIC proteins, which are known to function as ion channels when integrated into membranes, also demonstrate monothiol or dithiol (in the case of CLICs 2 and 3) glutaredoxin-like enzymatic activity when in their soluble form. This supports an additional role for the CLIC proteins in the cellular processes of detoxification and oxidoreduction. Furthermore, this PhD study provides direct evidence for the first time of the role of CLIC proteins to protect cells against oxidative stress. This also suggests that the enzymatic properties of the soluble CLICs may in turn also control the function of the membrane channel form.

1. 8 CLICs and their role in cancer

The CLICs 1, 3, 4 have been shown, in one way or another, to contribute to the initiation and/or progression of cancer. The following section will focus on what is currently known about the roles played by individual CLICs in various cancers.

CLIC1 is expressed in several cancers including ovarian carcinoma (100), hepatocellular carcinoma (101), high grade gliomas (101), human breast ductal carcinoma (102), gastric cancers (103), gallbladder metastasis (104) and nasopharyngeal carcinoma (105). In 2007 by Chen et al. (103), CLIC1 was found to be expressed in liver cancer and it was thought to alter cell division and/or apoptotic signalling, resulting in cellular transformation. CLIC1 was found to be upregulated in mouse hepatocellular carcinoma and this was further associated with cell migration and invasion (106, 107). The presence of CLIC1 in cancer cells has been associated with increased ROS production by NADPH oxidase, and this can lead to CLIC1 translocation as well as alterations to chloride fluxes (108). ROS levels are known to be fundamental for cell cycle progression. Therefore, at high ROS levels CLIC1 has the potential to insert into the membrane, or conversely CLIC1 insertion into the membrane is associated with increased ion flux which keeps ROS levels high. This could promote cell cycle progression (109).

CLIC1 has been suggested to be important in the development of glioblastoma. CLIC1 is highly expressed at the mRNA and protein level in glioblastoma, and its expression is upregulated in high grade tumours compared to low grade tumours (101, 110). In addition, silencing of CLIC1 in cancer stem cells injected into immune deficient mice reduced their self-renewal capacity as well as their proliferation and migration (109).

CLIC3 is highly expressed in tumour cells of Estrogen Receptor ER-negative breast cancers and in pancreatic adenocarcinomas, with high CLIC3 levels associated with reduced survival in these cancer types (111, 112). These same studies found that CLIC3's association with tumour aggressiveness is likely attributable to its ability to control the recycling of lysosomally-targeted α5β1 integrin and MT1-MMP back to the plasma membrane. Since then, other studies have been published associating CLIC3 with renal carcinoma and bladder cancer. In bladder cancer an antiangiogenic peptide CLT1 forms

complexes with fibronectin, killing proliferating cells through the α5β1 and CLIC3 pathway (113). The other study indicated that the plasma membrane sialidase NEU3 regulates the malignancy of renal carcinoma cells by controlling β1 integrin internalisation and recycling. Silencing of NEU3 upregulates the Ras-related protein RAB25. RAB25 directs internalised integrins to lysosomes. NEU3 silencing also downregulates CLIC3 which induces the recycling of internalised integrins to the plasma membrane. Therefore, silencing of NEU3 increased the β1 integrin endocytosis, but blocked recycling and thus reduced β1 levels at the plasma membrane (114).

CLIC4 has been shown to be present in the tumour profiling of glioma (115), melanoma (116), uterine leiomyoma (117) and bladder cancer (118). CLIC4 expression varied considerably in these tumours. Moreover, CLIC4 expression has been associated with a poor prognosis in colon cancer, and it is expressed in colon cancer stem cells (119). Furthermore, CLIC4 has been identified as a circulating biomarker in patients with ovarian carcinoma (120) and has been shown to be released in exosomes from ovarian cancer cells (121, 122), where the level of circulating exosomes in ovarian carcinoma was correlated with disease progression (123). Therefore, CLIC4 might be an ideal biomarker to measure tumour progression.

CLIC4 is highly expressed in the tumour cells and its functions as a tumour suppressor, but when it is expressed in the myofibroblast stroma, it is associated with tumour progression (124). CLIC4 downregulation in osteosarcoma cells in vitro and in vivo, its increased apoptosis and decreased cell proliferation, and this was enhanced following TNFα administration (124). In addition, other experiments showed that CLIC4 was lost in many tumours, but its highly expressed in the tumour stroma and led to malignant progression. CLIC4 is lost in the early evolution of tumours and the re-expression of exogenous CLIC4 in the tumour inhibited growth (125).

Furthermore, CLIC4 might act as a tumour suppressor, because its presence makes cells responsive to TGF-β-mediated growth inhibition, which in turn functions through keeping SMAD in a dephosphorylated state (126). From these studies, it appears clear that CLIC4 loss leads to tumour progression. Recently, other studies have shown that following TGF-β treatment, CLIC4 is one of the most upregulated proteins in myofibroblast stroma of breast cancer patients (127). Finally, CLIC4 has a major role in the angiogenesis and this may be important to the stromal contribution to tumour growth (128).

1. 9 Aims and Hypotheses of this Research Project

Members of the CLIC family have primarily been defined as intracellular ion channel proteins, which have been implicated in various biological processes, including cellular division (14), bone resorption (122), cell cycle regulation (37), p53-mediated apoptosis (39, 123-125), kidney function (48) and cancer-related processes such as cellular differentiation (126), tubulogenesis (48) and angiogenesis (127, 128).

In more recent times, the high structural homology between the CLIC proteins and Glutathione-S-Transferase super family of enzymes and in particular the GST-Omega-1 group has been revealed. Comparison of the two proteins, CLIC1 and GST-omega1 shows they both contain a single cysteine residue (Cys24 in CLIC1 and Cys32 in GSTO Ω1-1) in their active sites and have the ability to form a mixed disulfide bond with glutathione (47, 49, 81-83). Our group at UTS were the first to demonstrate via *in vitro* assays that members of the CLIC family specifically CLIC1, CLIC2 and CLIC4, in their soluble form, exhibit glutaredoxin-like enzymatic activity (97).

Given the current knowledge of the CLIC proteins' novel functions, beyond their well-established ion channel activity, along with their fairly ubiquitous expression across multiple cell types and evolutionary conservation across species, the current project hypothesis is that:

The CLIC proteins' play a fundamental cellular protective role against oxidative stress, which is derived from their oxidoreductase enzymatic activity.

Therefore, this research project aims to:

1- Demonstrate that like CLICs 1, 2 and 4, the protein CLIC3 also has intrinsic oxidoreductase enzymatic activity.

This aim is achieved by preparing pure recombinant CLIC(WT) proteins and testing their oxidoreductase activity using in vitro enzymatic assays typical of the glutaredoxin enzymes, (specifically the glutaredoxin HEDS (2-hydroxy ethyl disulfide) enzyme assay.

2- Determine the main structural residues that are involved in the enzymatic function demonstrated by the CLIC proteins, in particular for CLIC3, examine the critical active dithiol site cysteine residues and associated residues within the GST conserved GSH binding site motif.

This will be undertaken by using mutant forms of the various CLIC proteins and testing their in vitro activity in the HEDS assay.

3- Undertake cellular studies to investigate CLIC antioxidant functions. The oxidative stress tolerance of cells expressing recombinant CLIC proteins (WT) and mutant

forms will be examined. Exposing these cells to the oxidative agent (hydrogen peroxide) and determining if expression of CLIC proteins provides any protective advantage compared to control cells (105, 120).

E.coli bacterial cells transformed with genes coding for human CLIC protein will be used as the model system. The cells will be exposed to the oxidative agent hydrogen peroxide.

As previously mentioned, the CLIC family has been demonstrated to belong to the glutathione-S- transferase (GST) protein super-family (129). Members of the glutaredoxin and thioredoxin families act as detoxifying or stress response proteins by forming mixed disulfides between GSH and target proteins or low molecular weight thiols (a process known as glutathionylation) (110). It is now known that CLIC proteins can catalyse oxidoreductase reactions that are GSH dependent and thus similar to those catalysed by the GSTO1-1 and glutaredoxins, as such, their activity may extend to protein glutathionylation. Therefore, it is likely that the CLIC proteins can use their GSH-binding site to interact with specific protein targets in the cell (49) as well as using this same mechanism to regulate their own channel gating, once inserted into membranes (69). Therefore, this research project also aims to:

4- Determine if CLIC members can glutathionylate and/or deglutathionylate peptide / protein targets and seek to identify putative cellular protein targets.

In vitro fluorescence based assays using a glutathionylated and/or deglutathionylated synthetic peptide as a substrate will be employed. Identification of putative protein targets of CLIC proteins can be achieved by using transfected CHO-K1 cell lines

overexpressing CLIC1, and screening for changes in glutathionylation of proteins from whole cell lysates (WCLs) using western blots probed with an anti-GSH (attached to protein) antibody.

1. 10 References

1. **Nicolson GL.** 2014. The Fluid—Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40years. Biochimica et Biophysica Acta (BBA)-Biomembranes **1838:**1451-1466.
2. **Vellai T, Vida G.** 1999. The origin of eukaryotes: the difference between prokaryotic and eukaryotic cells. Proceedings of the Royal Society of London B: Biological Sciences **266:**1571-1577.
3. **Suzuki M, Morita T, Iwamoto T.** 2006. Diversity of Cl− channels. Cellular and molecular life sciences **63:**12-24.
4. **Golczak M, Kiser PD, Lodowski DT, Maeda A, Palczewski K.** 2010. Importance of membrane structural integrity for RPE65 retinoid isomerization activity. Journal of Biological Chemistry **285:**9667-9682.
5. **Jentsch TJ, Stein V, Weinreich F, Zdebik AA.** 2002. Molecular structure and physiological function of chloride channels. Physiological reviews **82:**503-568.
6. **Dworakowska½ B, Do³owy½ K.** 2000. Ion channels-related diseases.
7. **Dutzler R, Campbell EB, MacKinnon R.** 2003. Gating the selectivity filter in ClC chloride channels. Science **300:**108-112.
8. **Dutzler R, Campbell EB, Cadene M, Chait BT, MacKinnon R.** 2002. X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. Nature **415:**287-294.

9. **Al-Awqati Q.** 1995. Chloride channels of intracellular organelles. Current opinion in cell biology **7:**504-508.

10. **Edwards JC, Kahl CR.** 2010. Chloride channels of intracellular membranes. FEBS letters **584:**2102-2111.

11. **Jentsch TJ, Günther W.** 1997. Chloride channels: an emerging molecular picture. Bioessays **19:**117-126.

12. **Riordan JR, Rommens JM, Kerem B-s, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou J-L.** 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science **245:**1066-1073.

13. **al-Awqati Q, Barasch J, Landry D.** 1992. Chloride channels of intracellular organelles and their potential role in cystic fibrosis. The Journal of experimental biology **172:**245-266.

14. **Valenzuela SM, Martin DK, Por SB, Robbins JM, Warton K, Bootcov MR, Schofield PR, Campbell TJ, Breit SN.** 1997. Molecular cloning and expression of a chloride ion channel of cell nuclei. Journal of Biological Chemistry **272:**12575-12582.

15. **Stauber T, Weinert S, Jentsch TJ.** 2012. Cell biology and physiology of CLC chloride channels and transporters. Comprehensive Physiology.

16. **Jentsch TJ.** 2008. CLC chloride channels and transporters: from genes to protein structure, pathology and physiology. Critical reviews in biochemistry and molecular biology **43:**3-36.

17. **Uchida S.** 1999. Physiological role of CLC-K1 chloride channel in the kidney. Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association-European Renal Association **15:**14-15.

18. **Christensen EI, Devuyst O, Dom G, Nielsen R, Van Der Smissen P, Verroust P, Leruth M, Guggino WB, Courtoy PJ.** 2003. Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. Proceedings of the National Academy of Sciences **100:**8472-8477.

19. **Stobrawa SM, Breiderhoff T, Takamori S, Engel D, Schweizer M, Zdebik AA, Bösl MR, Ruether K, Jahn H, Draguhn A.** 2001. Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. Neuron **29:**185-196.

20. **Hara-Chikuma M, Wang Y, Guggino SE, Guggino WB, Verkman A.** 2005. Impaired acidification in early endosomes of ClC-5 deficient proximal tubule. Biochemical and biophysical research communications **329:**941-946.

21. **Koch MC, Steinmeyer K, Lorenz C, Ricker K, Wolf F, Otto M, Zoll B, Lehmann-Horn F, Grzeschik K-H, Jentsch TJ.** 1992. The skeletal muscle chloride channel in dominant and recessive human myotonia. Science **257:**797-800.

22. **Lloyd SE, Günther W, Pearce SH, Thomson A, Bianchi ML, Bosio M, Craig IW, Fisher SE, Scheinman SJ, Wrong O.** 1997. Characterisation of renal chloride channel, CLCN5, mutations in hypercalciuric nephrolithiasis (kidney stones) disorders. Human molecular genetics **6:**1233-1239.

23. **Xiong H, Li C, Garami E, Wang Y, Ramjeesingh M, Galley K, Bear C.** 1999. ClC-2 activation modulates regulatory volume decrease. The Journal of membrane biology **167:**215-221.

24. **Franciolini F, Nonner W.** 1994. Anion-cation interactions in the pore of neuronal background chloride channels. The Journal of general physiology **104:**711-723.

25. **Puljak L, Kilic G.** 2006. Emerging roles of chloride channels in human diseases. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease **1762:**404-413.

26. **Rinke I, Artmann J, Stein V.** 2010. ClC-2 voltage-gated channels constitute part of the background conductance and assist chloride extrusion. The Journal of Neuroscience **30:**4776-4786.

27. **Gyömörey K, Yeger H, Ackerley C, Garami E, Bear CE.** 2000. Expression of the chloride channel ClC-2 in the murine small intestine epithelium. American Journal of Physiology-Cell Physiology **279:**C1787-C1794.

28. **Duan D, Winter C, Cowley S, Hume JR, Horowitz B.** 1997. Molecular identification of a volume-regulated chloride channel. Nature **390:**417-421.

29. **Matsuda JJ, Filali MS, Moreland JG, Miller FJ, Lamb FS.** 2010. Activation of swelling-activated chloride current by tumor necrosis factor-α requires ClC-3-dependent endosomal reactive oxygen production. Journal of Biological Chemistry **285:**22864-22873.

30. **Hartzell C, Putzier I, Arreola J.** 2005. Calcium-activated chloride channels. Annu Rev Physiol **67:**719-758.

31. **Nilius B, Droogmans G.** 2003. Amazing chloride channels: an overview. Acta physiologica Scandinavica **177:**119-147.

32. **Waldegger S, Jentsch TJ.** 2000. From tonus to tonicity physiology of ClC chloride channels. Journal of the American Society of Nephrology **11:**1331-1339.

33. **Stauber T, Jentsch TJ.** 2013. Chloride in vesicular trafficking and function. Annual review of physiology **75:**453-477.

34. **Tomaskova Z, Ondrias K.** 2010. Mitochondrial chloride channels–What are they for? FEBS letters **584:**2085-2092.

35. **Duncan RR, Westwood PK, Boyd A, Ashley RH.** 1997. Rat brain p64H1, expression of a new member of the p64 chloride channel protein family in endoplasmic reticulum. Journal of Biological Chemistry **272:**23880-23886.

36. **Chuang J-Z, Milner TA, Zhu M, Sung C-H.** 1999. A 29 kDa intracellular chloride channel p64H1 is associated with large dense-core vesicles in rat hippocampal neurons. The Journal of neuroscience **19:**2919-2928.

37. **Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Warton K, Musgrove EA, Campbell TJ, Breit SN.** 2000. The nuclear chloride ion channel NCC27 is involved in regulation of the cell cycle. The Journal of physiology **529:**541-552.

38. **Tilley SJ, Saibil HR.** 2006. The mechanism of pore formation by bacterial toxins. Current opinion in structural biology **16:**230-236.

39. **Fernández-Salas E, Sagar M, Cheng C, Yuspa SH, Weinberg WC.** 1999. p53 and tumor necrosis factor α regulate the expression of a mitochondrial chloride channel protein. Journal of Biological Chemistry **274:**36488-36497.

40. **Redhead C, Sullivan S, Koseki C, Fujiwara K, Edwards J.** 1997. Subcellular distribution and targeting of the intracellular chloride channel p64. Molecular biology of the cell **8:**691-704.

41. **Edwards JC.** 1999. A novel p64-related Cl− channel: subcellular distribution and nephron segment-specific expression. American Journal of Physiology-Renal Physiology **276:**F398-F408.

42. **Cromer BA, Morton CJ, Board PG, Parker MW.** 2002. From glutathione transferase to pore in a CLIC. European Biophysics Journal **31:**356-364.

43. **Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, Valenzuela SM, Mazzanti M, Brown LJ, Breit SN.** 2010. The enigma of the

CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? FEBS letters **584:**2093-2101.

44. **Singh H.** 2010. Two decades with dimorphic chloride intracellular channels (CLICs). FEBS letters **584:**2112-2121.

45. **Dulhunty A, Gage P, Curtis S, Chelvanayagam G, Board P.** 2001. The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. Journal of Biological Chemistry **276:**3319-3323.

46. **Littler DR, Harrop SJ, Brown LJ, Pankhurst GJ, Mynott AV, Luciani P, Mandyam RA, Mazzanti M, Tanda S, Berryman MA.** 2008. Comparison of vertebrate and invertebrate CLIC proteins: The crystal structures of Caenorhabditis elegans EXC-4 and Drosophila melanogaster DmCLIC. Proteins: Structure, Function, and Bioinformatics **71:**364-378.

47. **Littler DR, Harrop SJ, Fairlie WD, Brown LJ, Pankhurst GJ, Pankhurst S, DeMaere MZ, Campbell TJ, Bauskin AR, Tonini R.** 2004. The intracellular chloride ion channel protein CLIC1 undergoes a redox-controlled structural transition. Journal of Biological Chemistry **279:**9298-9305.

48. **Berry KL, Bülow HE, Hall DH, Hobert O.** 2003. A C. elegans CLIC-like protein required for intracellular tube formation and maintenance. science **302:**2134-2137.

49. **Harrop SJ, DeMaere MZ, Fairlie WD, Reztsova T, Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Jankova L, Warton K.** 2001. Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-Å resolution. Journal of Biological Chemistry **276:**44993-45000.

50. **Berry KL, Hobert O.** 2006. Mapping functional domains of chloride intracellular channel (CLIC) proteins in vivo. Journal of molecular biology **359:**1316-1333.

51. **Elter A, Hartel A, Sieben C, Hertel B, Fischer-Schliebs E, Lüttge U, Moroni A, Thiel G.** 2007. A plant homolog of animal chloride intracellular channels (CLICs) generates an ion conductance in heterologous systems. Journal of Biological Chemistry **282:**8786-8792.

52. **Guex N, Peitsch MC.** 1997. SWISS-MODEL and the Swiss-Pdb Viewer: an environment for comparative protein modeling. electrophoresis **18:**2714-2723.

53. **Stoychev SH, Nathaniel C, Fanucchi S, Brock M, Li S, Asmus K, Woods Jr VL, Dirr HW.** 2009. Structural Dynamics of Soluble Chloride Intracellular Channel Protein CLIC1 Examined by Amide Hydrogen− Deuterium Exchange Mass Spectrometry. Biochemistry **48:**8413-8421.

54. **Lacroix E, Viguera AR, Serrano L.** 1998. Elucidating the folding problem of α-helices: local motifs, long-range electrostatics, ionic-strength dependence and prediction of NMR parameters. Journal of molecular biology **284:**173-191.

55. **Averaimo S, Abeti R, Savalli N, Brown LJ, Curmi PM, Breit SN, Mazzanti M.** 2013. Point mutations in the transmembrane region of the CLIC1 ion channel selectively modify its biophysical properties. PloS one **8:**e74523.

56. **Fanucchi S, Adamson RJ, Dirr HW.** 2008. Formation of an Unfolding Intermediate State of Soluble Chloride Intracellular Channel Protein CLIC1 at Acidic pH†. Biochemistry **47:**11674-11681.

57. **Carattino MD, Sheng S, Kleyman TR.** 2005. Mutations in the pore region modify epithelial sodium channel gating by shear stress. Journal of Biological Chemistry **280:**4393-4401.

58. **Ramsey IS, Moran MM, Chong JA, Clapham DE.** 2006. A voltage-gated proton-selective channel lacking the pore domain. Nature **440:**1213-1216.

59. **Parker MW, Feil SC.** 2005. Pore-forming protein toxins: from structure to function. Progress in biophysics and molecular biology **88:**91-142.

60. **Parker MW, Pattus F.** 1993. Rendering a membrane protein soluble in water: a common packing motif in bacterial protein toxins. Trends in biochemical sciences **18:**391-395.

61. **Thuduppathy GR, Hill RB.** 2006. Acid destabilization of the solution conformation of Bcl-XL does not drive its pH-dependent insertion into membranes. Protein science **15:**248-257.

62. **Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M, Reed JC.** 1997. Channel formation by antiapoptotic protein Bcl-2. Proceedings of the National Academy of Sciences **94:**5113-5118.

63. **Thuduppathy GR, Craig JW, Kholodenko V, Schon A, Hill RB.** 2006. Evidence that membrane insertion of the cytosolic domain of Bcl-X L is governed by an electrostatic mechanism. Journal of molecular biology **359:**1045-1058.

64. **García-Sáez AJ, Mingarro I, Pérez-Payá E, Salgado J.** 2004. Membrane-insertion fragments of Bcl-xL, Bax, and Bid. Biochemistry **43:**10930-10943.

65. **Ashley R.** 2003. Challenging accepted ion channel biology: p64 and the CLIC family of putative intracellular anion channel proteins (Review). Molecular membrane biology **20:**1-11.

66. **Murzin AG.** 2008. Metamorphic proteins. SCIENCE-NEW YORK THEN WASHINGTON- **320:**1725.

67. **Goodchild SC, Howell MW, Cordina NM, Littler DR, Breit SN, Curmi PM, Brown LJ.** 2009. Oxidation promotes insertion of the CLIC1 chloride

intracellular channel into the membrane. European Biophysics Journal **39:**129-138.

68. **Goodchild SC, Howell MW, Littler DR, Mandyam RA, Sale KL, Mazzanti M, Breit SN, Curmi PM, Brown LJ.** 2010. Metamorphic response of the CLIC1 chloride intracellular ion channel protein upon membrane interaction. Biochemistry **49:**5278-5289.

69. **Singh H, Ashley RH.** 2006. Redox regulation of CLIC1 by cysteine residues associated with the putative channel pore. Biophysical journal **90:**1628-1638.

70. **Landry D, Sullivan S, Nicolaides M, Redhead C, Edelman A, Field M, al-Awqati Q, Edwards J.** 1993. Molecular cloning and characterization of p64, a chloride channel protein from kidney microsomes. Journal of Biological Chemistry **268:**14948-14955.

71. **Singh H, Ashley RH.** 2007. CLIC4 (p64H1) and its putative transmembrane domain form poorly selective, redox-regulated ion channels. Molecular membrane biology **24:**41-52.

72. **Cromer BA, Gorman, M. A., Hansen, G., Adams, J. J., Coggan, M., Littler, D. R., ... Parker, M. W. .** 2007. Structure of the Janus Protein Human CLIC2. Journal of Molecular Biology, **374:** 719-731.

73. **Brumfield KM, Moroney JV, Moore TS, Simms TA, Donze D.** 2010. Functional characterization of the Chlamydomonas reinhardtii ERG3 ortholog, a gene involved in the biosynthesis of ergosterol. PLoS One **5:**e8659.

74. **Nezil FA, Bloom M.** 1992. Combined influence of cholesterol and synthetic amphiphillic peptides upon bilayer thickness in model membranes. Biophysical journal **61:**1176-1183.

75. **Tweten RK.** 2005. Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. Infection and immunity **73:**6199-6209.

76. **Valenzuela SM, Alkhamici H, Brown LJ, Almond OC, Goodchild SC, Carne S, Curmi PM, Holt SA, Cornell BA.** 2013. Regulation of the membrane insertion and conductance activity of the metamorphic chloride intracellular channel protein CLIC1 by cholesterol.

77. **Atkinson HJ, Babbitt PC.** 2009. Glutathione transferases are structural and functional outliers in the thioredoxin fold. Biochemistry **48:**11108-11116.

78. **Mittapalli O, Neal JJ, Shukle RH.** 2007. Tissue and life stage specificity of glutathione S-transferase expression in the Hessian fly, Mayetiola destructor: Implications for resistance to host allelochemicals. Journal of Insect Science **7:**20.

79. **Berryman M, Bretscher A.** 2000. Identification of a novel member of the chloride intracellular channel gene family (CLIC5) that associates with the actin cytoskeleton of placental microvilli. Molecular biology of the cell **11:**1509-1521.

80. **Sheehan D, MEADE G, Foley V, Dowd C.** 2001. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J **360:**1-16.

81. **Caccuri AM, Antonini G, Allocati N, Di Ilio C, De Maria F, Innocenti F, Parker MW, Masulli M, Bello ML, Turella P.** 2002. GSTB1-1 from Proteus mirabilis A SNAPSHOT OF AN ENZYME IN THE EVOLUTIONARY PATHWAY FROM A REDOX ENZYME TO A CONJUGATING ENZYME. Journal of Biological Chemistry **277:**18777-18784.

82. **Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV.** 2000. Identification,

characterization, and crystal structure of the Omega class glutathione transferases. Journal of Biological Chemistry **275:**24798-24806.

83. **Rossjohn J, Polekhina G, Feil SC, Allocati N, Masulli M, Di Ilio C, Parker MW.** 1998. A mixed disulfide bond in bacterial glutathione transferase: functional and evolutionary implications. Structure **6:**721-734.

84. **Oakley AJ, Rossjohn J, Lo Bello M, Caccuri AM, Federici G, Parker MW.** 1997. The three-dimensional structure of the human Pi class glutathione transferase P1-1 in complex with the inhibitor ethacrynic acid and its glutathione conjugate. Biochemistry **36:**576-585.

85. **Dalle-Donne I, Milzani A, Gagliano N, Colombo R, Giustarini D, Rossi R.** 2008. Molecular Mechanisms and Potential Clinical Significance of S -Glutathionylation, vol 10.

86. **Meyer Y, Buchanan BB, Vignols F, Reichheld J-P.** 2009. Thioredoxins and glutaredoxins: unifying elements in redox biology. Annual review of genetics **43:**335-367.

87. **Mieyal JJ, Gallogly, M. M., Qanungo, S., Sabens, E. A., & Shelton, M. D. .** 2008. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. . Antioxidants and Redox Signaling, **10** 1941-1988.

88. **Lin MTaB, M.F. .** 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature, **443,:**787-795.

89. **Holmgren A.** 1976. Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. Proceedings of the National Academy of Sciences **73:**2275-2279.

90. **Holmgren A.** 1983. Enzymatic reduction-oxidation of protein disulfides by thioredoxin. Methods in enzymology **107:**295-300.

91. **Wells WW, Yang Y, Deits TL, Gan ZR.** 1993. Thioltransferases. Advances in Enzymology and Related Areas of Molecular Biology, Volume 66**:**149-201.

92. **Holmgren A.** 1979. Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli. Journal of Biological Chemistry **254:**3664-3671.

93. **Holmgren A.** 1995. Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. Structure **3:**239-243.

94. **Lillig CH, Berndt C, Holmgren A.** 2008. Glutaredoxin systems. Biochimica et Biophysica Acta (BBA)-General Subjects **1780:**1304-1317.

95. **Littler D, Brown L, Breit S, Perrakis A, Curmi P.** 2010. Structure of human CLIC3 at 2 Å resolution. Proteins: Structure, Function, and Bioinformatics **78:**1594-1600.

96. **Littler DR, Assaad NN, Harrop SJ, Brown LJ, Pankhurst GJ, Luciani P, Aguilar MI, Mazzanti M, Berryman MA, Breit SN.** 2005. Crystal structure of the soluble form of the redox-regulated chloride ion channel protein CLIC4. FEBS Journal **272:**4996-5007.

97. **Al Khamici H, Brown LJ, Hossain KR, Hudson AL, Sinclair-Burton AA, Ng JPM, Daniel EL, Hare JE, Cornell BA, Curmi PM.** 2015. Members of the Chloride Intracellular Ion Channel Protein Family Demonstrate Glutaredoxin-Like Enzymatic Activity. PloS one **10:**e115699.

98. **Tang Z-X, Yang H-L.** 2013. Functional divergence and catalytic properties of dehydroascorbate reductase family proteins from Populus tomentosa. Molecular biology reports **40:**5105-5114.

99. **Lallement P-A, Brouwer B, Keech O, Hecker A, Rouhier N.** 2014. The still mysterious roles of cysteine-containing glutathione transferases in plants. Frontiers in pharmacology **5**.

100. **Averaimo S, Milton RH, Duchen MR, Mazzanti M.** 2010. Chloride intracellular channel 1 (CLIC1): Sensor and effector during oxidative stress. FEBS letters **584:**2076-2084.

101. **Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, Choi AM.** 2007. Mechanisms of cell death in oxidative stress. Antioxidants & redox signaling **9:**49-89.

102. **Biswas S, Chida AS, Rahman I.** 2006. Redox modifications of protein–thiols: emerging roles in cell signaling. Biochemical pharmacology **71:**551-564.

103. **Björnstedt M, Kumar S, Holmgren A.** 1994. Selenite and selenodiglutathione: reactions with thioredoxin systems. Methods in enzymology **252:**209-219.

104. **Das AK, Hummel BC, Gleason FK, Holmgren A, Walfish PG.** 1988. Bacterial and mammalian thioredoxin systems activate iodothyronine 5'-deiodination. Biochemistry and Cell Biology **66:**460-464.

105. **Cotgreave IA, Gerdes RG.** 1998. Recent trends in glutathione biochemistry—glutathione–protein interactions: a molecular link between oxidative stress and cell proliferation? Biochemical and biophysical research communications **242:**1-9.

106. **Bandyopadhyay S, Starke DW, Mieyal JJ, Gronostajski RM.** 1998. Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. Journal of Biological Chemistry **273:**392-397.

107. **Davis DA, Newcomb FM, Starke DW, Ott DE, Mieyal JJ, Yarchoan R.** 1997. Thioltransferase (glutaredoxin) is detected within HIV-1 and can regulate the activity of glutathionylated HIV-1 protease in vitro. Journal of Biological Chemistry **272:**25935-25940.

108. **Rodríguez-Manzaneque MT, Ros J, Cabiscol E, Sorribas A, Herrero E.** 1999. Grx5 Glutaredoxin plays a central role in protection against protein oxidative damage inSaccharomyces cerevisiae. Molecular and cellular biology **19:**8180-8190.

109. **Luikenhuis S, Perrone G, Dawes IW, Grant CM.** 1998. The yeast Saccharomyces cerevisiae contains two glutaredoxin genes that are required for protection against reactive oxygen species. Molecular Biology of the Cell **9:**1081-1091.

110. **Menon D, Board PG.** 2013. A role for glutathione transferase Omega 1 (GSTO1-1) in the glutathionylation cycle. Journal of Biological Chemistry **288:**25769-25779.

111. **Ruoppolo M, Lundström-Ljung J, Talamo F, Pucci P, Marino G.** 1997. Effect of glutaredoxin and protein disulfide isomerase on the glutathione-dependent folding of ribonuclease A. Biochemistry **36:**12259-12267.

112. **Yoshitake S, Nanri H, Fernando MR, Minakami S.** 1994. Possible differences in the regenerative roles played by thioltransferase and thioredoxin for oxidatively damaged proteins. Journal of biochemistry **116:**42-46.

113. **Hernandez-Fernaud JR, Ruengeler E, Casazza A, Neilson LJ, Pulleine E, Santi A, Ismail S, Lilla S, Dhayade S, MacPherson IR.** 2017. Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity. Nature communications **8:**14206.

114. **Jiang L, Phang JM, Yu J, Harrop SJ, Sokolova AV, Duff AP, Wilk KE, Alkhamici H, Breit SN, Valenzuela SM.** 2014. CLIC proteins, ezrin, radixin, moesin and the coupling of membranes to the actin cytoskeleton: a smoking gun? Biochimica et Biophysica Acta (BBA)-Biomembranes **1838:**643-657.

115. **Chrestensen CA, Eckman CB, Starke DW, Mieyal JJ.** 1995. Cloning, expression and characterization of human thioltransferase (glutaredoxin) in E. coli. FEBS letters **374:**25-28.

116. **Ghezzi P.** 2005. Oxidoreduction of protein thiols in redox regulation. Biochemical Society Transactions **33:**1378-1381.

117. **Kim Y-S, Kim I-S, Bae M-J, Choe Y-H, Kim Y-H, Park H-M, Kang H-G, Yoon H-S.** 2013. Homologous expression of cytosolic dehydroascorbate reductase increases grain yield and biomass under paddy field conditions in transgenic rice (Oryza sativa L. japonica). Planta **237:**1613-1625.

118. **Yin L, Wang S, Eltayeb AE, Uddin MI, Yamamoto Y, Tsuji W, Takeuchi Y, Tanaka K.** 2010. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta **231:**609-621.

119. **Do H, Kim I-S, Jeon BW, Lee CW, Park AK, Wi AR, Shin SC, Park H, Kim Y-S, Yoon H-S.** 2016. Structural understanding of the recycling of oxidized ascorbate by dehydroascorbate reductase (OsDHAR) from Oryza sativa L. japonica. Scientific reports **6:**19498.

120. **Jornstedt MB, Kumar S, Holmgren A.** 1995. [22] Selenite and selenodiglutathione: Reactions with thioredoxin systems. Methods in enzymology **252:**209-219.

121. **Rouhier N, Vlamis-Gardikas A, Lillig CH, Berndt C, Schwenn J-D, Holmgren A, Jacquot J-P.** 2003. Characterization of the redox properties of poplar glutaredoxin. Antioxidants and Redox Signaling **5:**15-22.

122. **Schlesinger PH, Blair HC, Teitelbaum SL, Edwards JC.** 1997. Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. Journal of Biological Chemistry **272:**18636-18643.

123. **Suh KS, Mutoh M, Gerdes M, Yuspa SH.** CLIC4, an intracellular chloride channel protein, is a novel molecular target for cancer therapy, p 105. *In* (ed), Nature Publishing Group,

124. **Fernández-Salas E, Suh KS, Speransky VV, Bowers WL, Levy JM, Adams T, Pathak KR, Edwards LE, Hayes DD, Cheng C.** 2002. mtCLIC/CLIC4, an organellular chloride channel protein, is increased by DNA damage and participates in the apoptotic response to p53. Molecular and cellular biology **22:**3610-3620.

125. **Suh KS, Mutoh M, Nagashima K, Fernandez-Salas E, Edwards LE, Hayes DD, Crutchley JM, Marin KG, Dumont RA, Levy JM.** 2004. The organellular chloride channel protein CLIC4/mtCLIC translocates to the nucleus in response to cellular stress and accelerates apoptosis. Journal of Biological Chemistry **279:**4632-4641.

126. **Suh KS, Mutoh M, Mutoh T, Li L, Ryscavage A, Crutchley JM, Dumont RA, Cheng C, Yuspa SH.** 2007. CLIC4 mediates and is required for Ca2+-induced keratinocyte differentiation. Journal of cell science **120:**2631-2640.

127. **Tung JJ, Kitajewski J.** 2010. Chloride intracellular channel 1 functions in endothelial cell growth and migration. J Angiogenes Res **2:**23.

128. **Tung JJ, Hobert O, Berryman M, Kitajewski J.** 2009. Chloride intracellular channel 4 is involved in endothelial proliferation and morphogenesis in vitro. Angiogenesis **12:**209-220.

129. **Pongstaporn W, Rochanawutanon M, Wilailak S, Linasamita V, Weerakiat S, Petmitr S.** 2006. Genetic alterations in chromosome 10q24. 3 and glutathione S-transferase omega 2 gene polymorphism in ovarian cancer. breast cancer **15:**16.

Chapter 2

Functional Studies to Confirm the Predicted Glutaredoxin-Like Enzymatic Activity of CLIC3

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Functional Studies to Confirm the Predicted Glutaredoxin-Like Enzymatic Activity of CLIC3

Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity

This chapter describes work that demonstrates for the first time the chloride intracellular ion channel protein CLIC3 also functions as an oxidoreductase enzyme in a manner similar to the chloride intracellular ion channel protein family members CLIC1, 2 and 4. This study formed part of a larger project undertaken in collaboration with the cancer research team at the Beatson Institute, University of Glasgow, UK. The project results were jointly published in the journal, *Nature Communications*. Full reference shown below:

Juan R. Hernandez-Fernaud, Elena Ruengeler, Andrea Casazza, Lisa J. Neilson, Ellie Pulleine, Alice Santi, Shehab Ismail, Sergio Lilla, Sandeep Dhayade, Iain R. MacPherson, Iain McNeish, Darren Ennis, **Hala Ali**, Fernanda G. Kugeratski1, Heba Al Khamici, Maartje van den Biggelaar, Peter V.E. van den Berghe, Catherine Cloix, Laura McDonald, David Millan, Aoisha Hoyle, Anna Kuchnio, Peter Carmeliet, Stella M. Valenzuela, Karen Blyth, Huabing Yin, Massimiliano Mazzone, Jim C. Norman & Sara Zanivan (2017) **Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity.** *Nature Communications*. 8:14206. DOI:10.1038/ncomms14206. www.nature.com/naturecommunications.

2. 1 Introduction

Living cells require energy to function properly under continuously fluctuating physiological conditions (1). The cells maintain optimal metabolic conditions by continuously adjusting their metabolism to these dynamic changes (2). Oxidation of nutrients, such as glucose, fatty acids and amino acids, produces cellular energy in the form of adenosine triphosphate, a main chemical energy carrier in all cells (3). Glycolysis is a central carbon metabolic pathway in nearly all organisms, in which cellular energy is generated by the degradation of glucose into pyruvate or lactate (3). Moreover, glycolysis provides vitally important precursors for macromolecular synthesis.

In an aerobic environment, pyruvate molecules are transported to mitochondria and are completely oxidized to carbon dioxide and water through the citric acid cycle and the electron transport system with the release of energy. Under anaerobic conditions, however, pyruvate is converted to lactate in order to regenerate oxidized nicotinamide adenine dinucleotide (NAD+), a molecule necessary to continue glycolysis. Glycolysis must be tightly regulated to maintain cellular metabolic balance and to optimize the production of energy and essential cellular functions.

Although oxygen is essential for life, the normal cellular metabolic processes lead to the generation of reactive oxygen species (ROS) which can in turn damage cellular components (4). ROS may occur in the form of either free radicals, such as the highly reactive hydroxyl radical (OH•) or they may be nonradicals such as hydrogen peroxide (H_2O_2) (5-7). These ROS are the major causes of oxidative stress which usually occurs as a result of an imbalance

between ROS production and the scavenging activity of the cellular antioxidant defence mechanisms (6).

A major intracellular antioxidant defense system is the non-protein thiol tripeptide glutathione (GSH). Glutathione is an antioxidant consisting of γ-glutamyl-cysteinyl-glycine synthesized by γ -glutamylcysteinyl synthetase (8, 9). The glutathione redox cycle involves cycling between the reduced form of glutathione (GSH) and the oxidized glutathione (GSSG) through reduction of GSSG by the enzyme glutathione reductase (10, 11), with the GSH/GSSG ratio being a good indicator of intracellular redox status (12). Several other enzymes use GSH to detoxify radical species, including glutathione peroxidases and glutathione S-transferases (13).

Thioredoxins as well as glutaredoxins have been conserved throughout evolution and show significant functional redundancy. They are the key components of the thiol redox systems that are involved in protecting cells against ROS (7, 14). Thioredoxins and glutaredoxins are regarded as general disulfide reductases that also function in maintaining the thiol redox homeostasis of the cell (15). Thioredoxins and glutaredoxins play an important role in removing ROS from cells thus protecting the cell against oxidative stress (16). The activities of these redoxins will be discussed in greater detail below.

2. 2 Cellular Protective Mechanisms

2.2. 4 Thioredoxins

Thioredoxins (Trx) were discovered in 1964 as essential cofactors for ribonucleotide reductase (RNR) (17). Thioredoxin together with thioredoxin reductase (TrxR) make up the thioredoxin system, which is an essential regulator of the intracellular redox state.

Thioredoxin is present in numerous species and possesses a conserved active site that is characteristic of the thioredoxin superfamily (CxxC/S) (18).

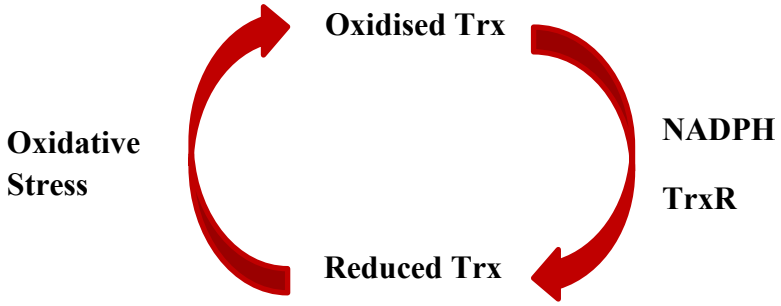

Figure 2. 1 Scheme of the thioredoxin antioxidant system in the presence of oxidative stress. *Oxidised thioredoxin (TRX) is reduced by thioredoxin reductase (TxrR) in the presence of NADPH and re-oxidised under oxidative stress conditions or during physiologically relevant reduction activities. This figure was adapted from (19).*

In the thioredoxin system, electrons are transferred from NADPH through the activity of thioredoxin reductase to the thioredoxin active site. Upon the reduction of a target protein, this active site becomes oxidised (20) and thioredoxin reductase then recycles the disulphide back into its dithiol form (18) (Figure 2.1). Thioredoxins play an important role in the defence against oxidative stress by providing the necessary electrons for the peroxiredoxins in the cell.

Interestingly, human Trx1 possesses three non-active site cysteine residues (Cys62, Cys69 and Cys 73) in addition to their active site which have been implicated in the regulation of the activity of this thioredoxin (21). Under oxidising conditions, a second intramolecular disulfide distinct from the active site disulfide bond has been observed in these thioredoxins (21, 22) which disrupts the interaction that occurs between thioredoxin

and its target proteins and inactivates Trx1 activity (21). These non-active site cysteines may also possess the ability to transfer electrons to peroxiredoxins (21). In summary, thioredoxins work together with the peroxiredoxins in antioxidant activity, where peroxiredoxins use electrons donated by the thioredoxin in scavenging intracellular ROS (7).

2.2. 5 Glutaredoxins

Glutaredoxin (Grx) is the most thoroughly characterized deglutathionylating enzyme (23, 24). Glutaredoxins were identified as GSH-dependent reductases when growth of a thioredoxin mutant of *E. coli* was observed suggesting that another protein was able to reduce ribonucleotide reductase (25). Glutaredoxins are evolutionarily conserved, heat-stable oxidoreductases with active sites which contain one or two cysteine residues (26). Depending on the number of cysteine residues at the CxxC/S active site, glutaredoxins can be separated into monothiol or dithiol type glutaredoxins (27, 28). The glutaredoxins can further be grouped based on their physiological roles, subcellular localisation and biochemical properties (28). They are structurally all members of the thioredoxin superfamily as they have a thioredoxin fold but are distinguishable from thioredoxins by their specificity for GSH (28).

The monothiol mechanism requires only the N-terminal cysteine residue for the reduction of the GSH mixed disulfides whereas the dithiol mechanism proposes the use of both of the active site cysteine residues to reduce glutathionylated substrates. Interestingly, the dithiol mechanism is also used for the reduction of low molecular weight and protein disulfide substrates such as ribonucleotide reductase (29-31), refer to Figure 2.2 below.

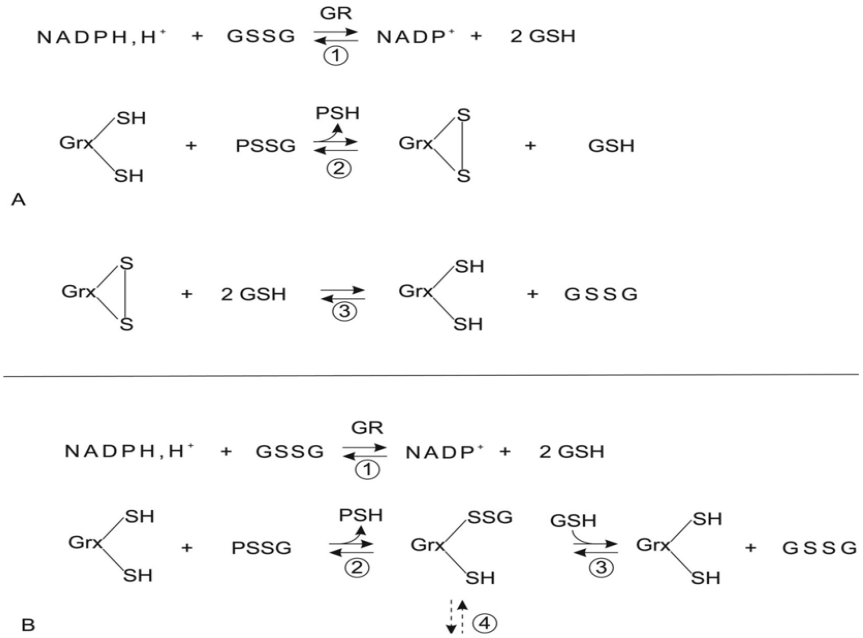

Figure 2. 2 Proposed mechanisms for glutaredoxin activity showing the deglutathionylation of glutathionylated protein (PSSG) mixed disulfides catalysed by glutaredoxins. *The dithiol mechanism (A) shows the reduction of the intramolecular disulfide (GrxSS) at the active site of the glutaredoxins by 2 GSH molecules forming glutathione disulfide (GSSG) and recycling the reduced glutaredoxin (Grx-S-H). The monothiol mechanism (B) shows the reduction of GrxSSG by GSH producing glutathione disulfide (GSSG) as the second product and recycling the reduced enzyme (Grx-S-H) is considered the rate-determining step. Shown in reaction 4 of this mechanism is a connected side reaction which includes the formation of the intramolecular disulfide (GrxSS) at the active site of the glutaredoxins* **(32).**

Thioredoxins as well as glutaredoxins (Grx) have been conserved throughout evolution and significant functional redundancy between these two systems has been recognised with regards to ribonucleotide reductase (RNR) which reduces ribonucleotides to deoxyribonucleotides (dNTPs), 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase and plasma GSH peroxidise activity (33). Normal aerobic growth is

independent of either the thioredoxin or glutaredoxin branch individually, but when both systems are inactivated, cells are unviable as these systems are essential for ribonucleotide reductase activity (34).

2.2. 6 Glutathione-S-Transferases

GST proteins can be divided into at least 12 classes of proteins. They are multifunctional enzymes that exist mostly in a dimeric form in the cytosol (35). GSTs are cytosolic proteins which can catalyse the conjugation of a tripeptide (glutamine, cysteine, and glycine) glutathione (GSH) to electrophilic regions of other molecules (36). This reaction can occur through activation of the thiol group of GSH, which allows noncovalent, but high-affinity binding to the substrate (37, 38). Therefore, like the thioredoxins, glutathione transferases catalyse reduction of cysteine using glutathione as a source of reducing equivalents (39). Several GST isoforms have also been reported to catalyze protein deglutathionylation (40). In each case, non-glutathionylated pro-substrates (e.g., HEDS, S-sulfocysteine) were used to assess deglutathionylation activity. To become glutathionyl mixed disulfides, these pro-substrates must first react with GSH, forming βME-SSG or CSSG (23, 41). Importantly, this assay cannot distinguish between catalysis of the glutathionylation step (RSSG formation, characteristic of a GST-mediated conjugation) and the deglutathionylation step (RSSG reduction, characteristic of a deglutathionylase such as Grx). Interestingly, Dal Monte et al. (1998) (42) observed that bovine lens GSTμ, catalyzes formation of βME-SSG from HEDS and GSH but has a negligible effect on the rate of βME-SSG deglutathionylation. Furthermore, when a protein-SSG substrate was used to test GST-mediated deglutathionylation, addition of GST to a reaction mixture containing Grx did not augment the deglutathionylation activity (43).

As described in Section 2.2.2, Grx is known as the most specific and efficient catalyst of deglutathionylation in mammalian cells. This catalytic activity has been demonstrated to regulate diverse cellular processes, including metabolism, calcium homeostasis, cytoskeletal reorganization, hypertrophy and inflammation (44).

2. 3 CLICs demonstrate structural and functional similarities to other GST superfamily members

The first GST enzymes identified were associated with xenobiotic metabolism. However, since whole genomes have been decoded, more and more proteins have been found to belong to the GST fold superfamily. This includes the identification of several other GSTs. Amongst the new additions, the CLIC protein family has now also been identified as belonging to the GST superfamily, which despite their weak sequence homology (15%) have strong structural and functional similarities in particular with the GST omega (GSTO) proteins (45). Structural analysis studies have provided strong evidence that the CLIC proteins should be considered part of the GST superfamily (37, 45).

A GST-fold is made up of two different domains. One of the domains is made up of the α-helical C-terminal. The second domain has been described as the N-terminal thioredoxin fold. Most of the thioredoxin proteins have an active site in which a redox-active cysteine is located. This specific site is not present in all GST proteins (36). As previously mentioned, the CLICs are comprised of a ~240 residue common cassette, that adopts a GST superfamily fold (37, 45). The CLIC proteins' globular form is comprised of an N terminal thioredoxin domain, which contains four β-strands in between three α-helices and is where the conserved glutaredoxin monothiol or dithiol motif is located (46). A conserved active site cysteine in the N-terminal domain is found in all CLIC proteins

(Cys24 in CLIC1). Structure / function studies confirm that the cysteine sits within the proteins' enzymatic active site. Furthermore, this active site cysteine likely becomes activated by the protein itself and thereafter is capable of forming disulfide bridges with GSH (47). Moreover, in CLICs 2 & 3 the 'active site cysteine' is adjacent to another cysteine, thus forming a di-cysteine (dithiol) motif (Cys-X-X-Cys).

Experiments by our group at UTS in which 2-hydroxyethyl disulphide (HEDS) was used as a substrate have recently demonstrated that CLICs 1, 2 and 4 have "glutaredoxin-like glutathione dependent oxidoreductase enzymatic activity" (48), which was abolished in CLIC1 following mutation of the putative 'active site cysteine'. Moreover, the indanyloxyacetic acid (IAA) and ethacrynic acid compound (IAA-94) and the drug A9C known to inhibit the CLIC1 ion channel also inhibited the glutathione-dependent oxidoreductase activity of CLIC1. Conversely, 4, 4'Di isothiocyano-2, 2'-stilbenedisulfonic acid (DIDS) had no effect on CLIC1's oxidoreductase activity (48).

The chloride intracellular channel protein 3 (CLIC3) was recently shown to be an abundant component of the Cancer-Associated Fibroblast secretome (CAFs) (49). Secreted CLIC3 was shown to promote invasive behavior of endothelial cells. This in turn was shown to drive angiogenesis and increased invasiveness of cancer cells both *in vivo* and in 3D cell culture models and it was found to require the presence of active transglutaminase-2 (TGM2) (49). As part of this thesis, CLIC3 was demonstrated to act as a glutathione-dependent oxidoreductase, which was used to support the above mentioned findings by our collaborators, who showed that it acts to enzymatically reduce TGM2 and thus regulates TGM2 binding to its cofactors (49). Our recent studies demonstrated for the first time that CLIC3 is also a GSH-dependent oxidoreductase, with a dithiol active site motif. This thesis describes these findings, which also confirmed the

critical active site was comprised of cysteines 22 and 25. In combination with the fact that CLIC3 controls the GSH-dependent reduction of TGM2 at specific cysteines, CLIC3 influences the binding of TGM2 to its regulatory cofactors (49).

In summary, it has become more apparent that CLIC proteins function as oxidoreductases. Experiments in which 2-hydroxyethyl disulphide was used as a substrate have recently indicated that CLIC1, 2 and 4 have glutaredoxin-like glutathione-dependent oxidoreductase enzymatic activity (48), which was inhibited by mutation of the putative active site cysteine. Moreover, the indanyloxyacetic acid (IAA) and ethacrynic acid compound (IAA-94) and the drug A9C that have been previously identified as CLIC binding molecules termed 'chloride channel blockers', also inhibit the glutathione-dependent oxidoreductase activity of CLIC1. In contrast, 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), a well-established chloride channel antagonist, which does not block CLIC1's channel activity, also had no effect on CLIC1's oxidoreductase activity (48) .

Given the recent work describing CLIC1's glutaredoxin- like, glutathione-dependent oxidoreductase enzymatic activity, and studies indicating the high level of structural homology between CLIC proteins, GST-Ω, Grxs and Trxs which are well-known enzymes, we proposed that CLIC3 would also function as an oxidoreductase enzyme (37, 47). This chapter describes the investigations undertaken to ascertain this putative enzymatic activity of the soluble form of CLIC3 using a set of assays typically designed to measure the activity of glutathione-dependent enzymes.

2. 4 Materials and Methods

2.4. 3 Chemicals and Reagents

All the chemicals used to perform the experiments in this research project are of high analytical grades. All buffers and solutions were prepared using demineralised, ultrapure water that was prepared with the Arium® Pro (Sartorius) water system. Ultrapure, demineralised water for aseptic preparation of solutions and media was sterilised before use by a steam autoclave (Systec DX-150). In addition, all antibiotics and solutions were sterilised by filtration using 0.22 μm Millex®GP filter sets (Millipore) and were stored in -20ºC until needed. The *Escherichia coli* BL21 (DE3) pLysS super-competent cells were purchased from New England Biolabs® Inc. (Ipswich, MA). The plasmid DNA QIAprep® Spin Miniprep Kit was purchased from QIAGEN (Australia).

All restriction enzymes used were purchased from New England Biolabs (NEB). Culture media, sterile PBS, antibiotics, were obtained from Life Technologies (Gibco). Chemicals used for treatment of mammalian cell lines were dissolved in cell culture grade DMSO (Sigma Aldrich). Cholesterol (Chol) was purchased from Sigma Aldrich (Australia). The following reagents used in HEDS enzyme assay were all purchased from Sigma Aldrich: glutathione reductase (GR) from yeast, reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 2hydroxyethyl disulphide (HEDS), indanyloxyacetic acid (IAA-94), Isopropyl-thio-β-D-galactopyranoside (IPTG), Dithiothreitol (DTT), tris-2-carboxyethyl-phosphine (TCEP) and thrombin from bovine plasma were purchased from Sigma Aldrich. HiPrep 16/60 Sephacryl S-100 HR GE Healthcare column (pre-packed gel filtration column) was purchased from VWR International (Queensland, Australia).

2.4. 4 Methods

2.4.2. 7 Bacterial Strains and Plasmids

All plasmids were maintained and manipulated in bacterial strain *Escherichia coli* BL21 (DE3). The *Escherichia coli* BL21 cells transformed with pET-28a plasmid containing the genetic sequence of the recombinant His-CLIC1 fusion protein was a gift from Dr S. N. Breit, Centre of Immunology, St. Vincent's hospital, University of New South Wales, Sydney Australia. Glycerol stocks of *Escherichia coli* BL21 (DE3) transformed with the CLIC1-pET-28a plasmid were streaked out on Luria broth/Kanamycin (LB/Kan) plates and grown overnight in LB media supplemented with a 30 μg/ml Kanamycin at 37°C. Clones were stored as 50% glycerol stocks at -80°C.

The pGEX-6P-1 plasmids containing the open reading frame for the proteins CLIC3-wt and CLIC3-Cys22A (Cysteine 22 mutated to Alanine) and CLIC3-Cys22,25A (Cysteine 22,25 mutated to Alanine) mutants were obtained from Dr Sara Zanivan, Cancer Research UK Beatson Institute, Glasgow G611BD, UK. These plasmids were transformed in bacterial strain *Escherichia coli* BL21 (DE3) and streaked out on Luria broth/Carbenicillin (LB/Car) plates and grown overnight in LB media supplemented with a 30 μg/ml Carbenicillin at 37°C. Transformed clones were stored as 50% glycerol stocks at -80°C.

The pGEX-4T plasmids containing the open reading frame for the proteins CLIC4-wt was obtained from (Prof Paul Curmi group, School of Physics, University of New South Wales, Sydney, Australia). The plasmid was transformed in bacterial strain *Escherichia coli* BL21 (DE3) and streaked out on Luria broth/Carbenicillin (LB/Car) plates and grown overnight in LB media supplemented with a 30 μg/ml Carbenicillin at 37°C. Transformed clones were stored as 50% glycerol stocks at -80°C.

For protein expression and purification purposes, respective clones were transformed into bacterial strain *Escherichia coli* BL-21 (DE3) and grown under Kanamycin/Carbenicillin selection.

2.4.2. 8 Plasmid DNA Preparation

Plasmid DNA was isolated from *E. coli* cells using a miniprep kit (QIAprep® Spin Miniprep Kit from QIAGEN, Pvt. Ltd., Australia). In brief, cells were harvested, and plasmids were extracted by alkaline lysis. Cells were re-suspended and lysed in 250 µl of sodium hydroxide containing Reagent P1 followed by protein denaturation with 250 µl of Reagent P2 containing SDS. The alkaline suspension was neutralized with 350 µl sodium acetate (Reagent P3). The precipitate was then separated from the supernatant by centrifuging the samples at 13000 x g for 10 minutes. The clear supernatant was transferred into Qiagen spin columns. After centrifugation at 13000 x g for 1 minute, DNA was retained on the spin column membrane which was then repeatedly washed with Ethanol. The membrane was incubated in 50 µl DNase free sterile water for a minute prior to elution by centrifugation. The purified plasmid was then quantified using a NanoDrop ND-1000 spectrophotometer.

2.4.2. 9 Restriction Enzyme Digests

Plasmid DNA (less than 1µg DNA) was double digested with restriction enzymes as per (NEB) manufacturer's instructions. The digestion reaction was set up in a suitable buffer, common to both restriction endonucleases. The reaction volume was made up with water to a total of 10 µl for all digestions. The reactions were incubated at 37°C for two hours. Final concentrations of enzymes, buffers and BSA were maintained at 1/10th the total volume.

2.4.2. 10 Transformation of plasmids into bacterial cells

Heat shock transformation was applied as previously described by Froger et al. (2007) (50). Ligation reactions were transformed by adding 2 µl of plasmids into 50 µl aliquots of competent *E.coli* BL21 (DE3) cells and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 45 seconds and immediately returned to ice for a further 5 minutes. The cells were then incubated for 1 hour at 37°C with shaking, following addition of 500 µl of LB media without antibiotics. Transformed cells were then plated onto LB plates containing ampicillin and incubated overnight at 37°C to allow cellular growth and stabilisation.

The mixture was centrifuged at 2500 rpm for 2 minutes. 100 µl of the culture was spread on LB-plates supplemented with Kanamycin/ Carbenicillin antibiotics according to the antibiotic resistance of transformed plasmids, then the plates incubated at 37°C overnight. A single colony was selected and cultured overnight at 37 °C in 100 ml 2x YT media. The plasmids of different CLICs proteins were isolated using the QIAprep® Spin Miniprep kit (QIAGEN).

2.4.2. 11 Nucleic Acid Quantification

Nucleic acid concentration and purity was determined using a spectrophotometer (Nanodrop Technology, Biolab). Concentration was measured in terms of the corresponding optical density (O.D.) at a wavelength of 260 nm. Purity of DNA/RNA was estimated by calculating 260/280. A ratio of approximately 1.8 is considered pure for double stranded DNA.

2.4.2. 12 Agarose Gel Electrophoresis

In this study the BIORAD Mini-Protean gel electrophoresis system (Biorad, Australia) were used to run the products of digested plasmids by restriction enzyme. Gels varied

from 0.8-1.5% in agarose concentration. Agarose (Sigma Aldrich, Australia) was dissolved in 1X of TBE buffer (Tric, Boric acid and Ethylenediaminetetraacetic acid EDTA) by gradual heating. The solution was allowed to cool to 50°-60°C before adding 0.02% Gel Red, a DNA binding dye that allows visualization of DNA by UV illumination. DNA samples were diluted with 6X loading dye in a 1:10 ratio prior to loading. The gels were run in the 1X TBE buffer that used to prepare the gel at 65V for approximately 1 hour.

2. 5 Expression and purification of recombinant CLIC proteins

2.5. 3 2xYT media for bacterial growth

We used the 2xYT media for the cultivation of bacteria *Escherichia coli* BL21 (DE3). This medium is composed of yeast extract (10 g), bacteriological tryptone (15 g) and NaCl (5 g) (Sigma Aldrich). These reagents were weighted, mixed and dissolved in sterile deionized water to a final volume of 1 L and subsequently autoclaved for 40 minutes at 1.5 kg f/cm^2 at 122 °C.

2.5. 4 Production of Recombinant CLIC Proteins

2.5.2. 14 Small Scale Culture Preparation

Six individual sterile conical flasks containing 20 ml. of 2xYT media and 20 µl of kanamycin antibiotic with stock concentration of 30 mg/ml (Sigma Aldrich) were inoculated with 10 µl of bacterial growth taken from glycerol stocks of *Escherichia coli* BL21 (DE3) cells (Stratagene, USA), transformed with the His-tagged pET-28a vector (Novagen) containing the coding sequence for either the protein CLIC1-wt and mutants CLIC1-Cys24S (Cysteine 24 mutated to Serine), CLIC1-29A (Arginine 29 mutated to

Alanine), CLIC1-37A (Lysine 37 mutated to Alanine) were used in order to express the recombinant protein. Furthermore, the GST-tagged pGEX-6P-1vector (New England Biolabs (NEB) containing the coding sequence for the protein CLIC3-wt, CLIC3-Cys22,25A (Cysteine 22,25 mutated to Alanine) and CLIC3-Cys22A (Cysteine 22 mutated to Alanine) were used in order to express the recombinant proteins. Additionally, the pGEX-2T GST-tagged containing the coding sequence for either the CLIC4-wt and Exc-4, were used to express the recombinant proteins. These small flasks were left to grow overnight in a shaking incubator set at 200 rpm, at 37°C. (Table 1.1)

Table 2. 1: A summary of the cDNA clones used to express recombinant human CLIC proteins in *Escherichia coli* BL21 (DE3) bacteria and differences in their G-site motifs.

CLIC cDNA Construct	***Molecular Weight (kDa)***	***Conserved G-site motif***	***Vector Plasmid Containing Open Reading Frame***	***Antibiotic Resistance***	***Gene Fusion System***
CLIC1WT	26.9	C-P-F-S	pET28a	Kanamycin	His-tagged
CLIC1 C 24 A	26.9	**A**^{24}-P-F-S	pET28a	Kanamycin	His-tagged
CLIC1 C 24 S	26.9	**S**^{24}-P-F-**S**	pET28a	Kanamycin	His-tagged
CLIC1 K 37 A	26.9	C-P-F-S	pET28a	Kanamycin	His-tagged
CLIC1 R 29 A	26.9	C-P-F-S	pET28a	Kanamycin	His-tagged
CLIC3 WT	26.7	C-P-S-C	pGEX-6P-1	Carbenicillin	GST-tagged
CLIC3 C22A	26.7	**A**^{22}-P-S-C	pGEX-6P-1	Carbenicillin	GST-tagged
CLIC3 C22A & C25A	26.7	**A**^{22}-P-S-**A**^{25}	pGEX-6P-1	Carbenicillin	GST-tagged
CLIC4 WT	28.6	C-P-F-S	pGEX-2T	Carbenicillin	GST-tagged
EXC-4	27	D-L-F-C	pGEX-2T	Carbenicillin	GST-tagged
HcTrx-5	19.8	C-R-S-C	pTrcHisB	Carbenicillin	His-tagged

2.5.2. 15 Large Scale Culture Preparation and Protein induction

Each individual large flask containing 350 ml of 2xYT media and 30 mg/ml kanamycin were inoculated with 20 ml. of small-scale bacterial cultures prepared as described above in Section 2.5.1.1. The upscaled flasks were left to grow in a shaking incubator at 200 rpm at 37°C for 1.5 hours. Subsequently, once the bacterial growth achieved an optical density (OD) at 600 nm value between 0.6-0.8, At that point, 1 ml of sample was taken as an uninduced control. Then 1 mM of IPTG (Isopropylthiogalactoside) was added to the media to induce the expression of the His-tagged proteins. Finally, the bacterial cultures were incubated at 20°C for a further 16 hours with shaking at 200rpm.

2.5.2. 16 Harvesting the induced Escherichia coli bacterial cells

The next day, the induced cells were harvested by centrifugation using High Speed Refrigerated Centrifuge (Hitachi CR22GIII) fitted with a rotor R13A rotor at 8000 rpm for 30 minutes at 4°C. After centrifugation, the bacterial pellets were collected and pooled in a 50ml falcon tube, the pellet was either stored at - 80°C for later use or resuspended in about 15 ml of lysing buffer (300 mM KCl, 50mM potassium phosphate buffer pH 8.0 and 5mM Imidazole). 1 mM protease inhibitor cocktail (2 mM AEBSF, 0.3 µM Aprotinin, 130 µM Bestatin, 1 mM EDTA, 14 µM E-64, 1 µM Leupeptin) was added fresh per litre of original culture and incubated at 4°C to initiate cell lysis.

2.5.2. 17 Lysing of Escherichia coli bacterial cells

The resuspended *E.coli* cells were subsequently sonicated on ice using the sonicator (Sonics and Materials/Vibra- Cell Ultrasonic Liquid Processor) for 15-20 seconds per cycle at 700 psi; until the viscosity of solution became greatly decreased. Afterwards, we

add a 20% (v/v) Triton X-100 solution (8 ml. of H2O added to 2 ml of Triton X-100). The 20% (v/v) Triton X-100 solution was prepared from the original stock of Triton X-100 and then 1 ml of the 20% Triton X-100 solution was added to 25 ml of bacterial homogenites. The sonicated cells were then collected and spun using at 10000 rpm for 40 minutes at 4°C. Then, the supernatant containing soluble proteins were transferred to a 50 ml. falcon tube and filtered through a 0.45 μm filter to remove any remaining cell debris and kept on ice until subjected to further purification.

2.5.2. 18 First Stage of Purification of CLIC1 using Ni^{2+} -NTA resin

The purification of recombinant His-tagged fusion proteins performed using the Histidine-tagging method. This high specific affinity chromatography method was designed using a specific ligand covalently immobilised on a suitable matrix (agarose). Only molecules in the mobile phase that have an affinity for the immobilised ligand will be bound to the column. This method is based on the selective interaction between soluble HisCLIC1 fusion protein (located in the supernatant of *Escherichia coli* BL21 (DE3) lysate) to Ni^{2+} ions covalently bonded to the immobilised nitrilotriacetic acid matrix (NTA), where Ni-NTA and His-CLIC1 are the immobilised ligand and the target molecule, respectively (Figure 2.3). After binding to the immobilised ligand, elution of the proteins was performed by introducing a competing agent such as imidazole or an additional metal chelating agent (EDTA). Purification and all experimental procedures were performed under reducing conditions, using 0.5 mM tris-2-carboxyethyl-phosphine (TCEP) to prevent oxidative dimerization of the protein (51).

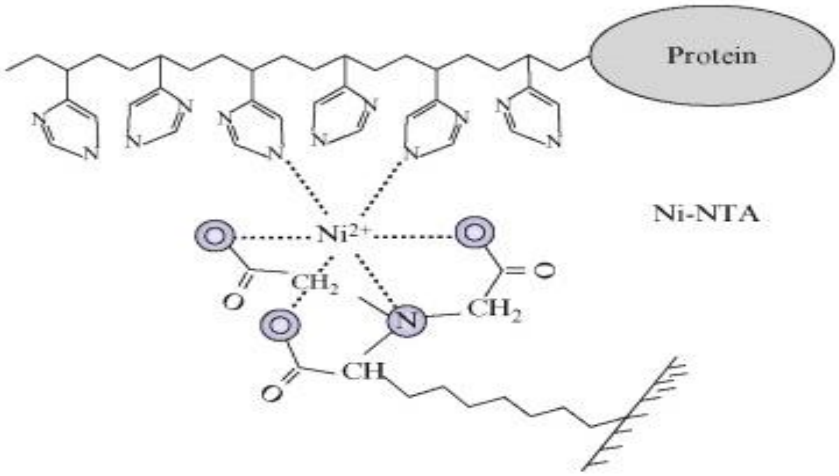

Figure 2. 3 Schematic diagram showing the complex formed between the poly-Histidine tagged protein and a Ni-NTA matrix (52).

A 2 ml Ni-NTA suspension (Bio-Rad) was packed in 10 ml Bio-Rad column and equilibrated at 20°C with 10-column volumes (CV) of Binding buffer, pH 8.0 (300 mM KCL, 50 mM KH2PO4, 10 mM Imidazole). The clarified lysate was loaded onto the column and incubated at 4°C for one hour with mild agitation to allow optimum binding between His-CLIC1 fusion protein and the Ni-NTA matrix. The soluble His-CLIC1 fusion protein in the supernatant of the *Escherichia coli* BL21 (DE3) lysate binds via the His to the nickel beads while the rest of the protein solution passes through the column. Hence, CLIC1-His fusion protein is isolated from other proteins produced by the *Escherichia coli* cells. The column was subsequently washed with 10-column volumes of the Binding buffers followed by Wash Buffer, pH 8.0 (300 mM KCL, 50 mM KH2PO4, 20 mM Imidazole) to remove any non-specifically bound proteins and any unbound His-CLIC1. Thereafter, a volume of 50 µl (1 U/ml) of bovine plasma thrombin per litre of cell culture, was added to the His-CLIC1 fusion protein and incubated at 4°C for at least 16 hours with mild agitation. The column was sealed, and digestion was allowed to occur on

a rotator for 16 hours at 4°C resulting in the cleavage of CLIC1 from the His tag and, therefore, CLIC1 was released from the matrix. The CLIC1 cleaved from His and the thrombin used for cleavage was collected from the column using Elution Buffer, pH 8.0 (300 mM KCL, 50 mM KH2PO4, 250 mM Imidazole) in fractions of 2 ml and pooled together in preparation. The column was regenerated with 0.5 M NaOH solution according to the manufacturer's instructions.

Eluted fractions were run on an SDS-PAGE gel and quantified by absorbance spectrometry at A280nm using a ND-1000 spectrophotometer (Nanodrop). The fractions containing CLIC1 were stored at -80°C to subject for further purification. Purification and all experimental procedures were performed under reducing conditions, using 1 mM Dithiothreitol (DTT) or 0.5 mM tris-2-carboxyethyl-phosphine (TCEP) to prevent oxidative dimerization of the protein (51).

2.5.2. 19 Preparation of recombinant CLIC1 mutants

Procedures for the expression, first stage of purification and quantification of recombinant CLIC1-Cys24S, CLIC1-R29A and CLIC1-K37A mutants followed the same methodology as described above in Section 2.5, for recombinant CLIC1.

2.5.2. 20 Preparation of recombinant CLIC3, CLIC3 mutants, CLIC4 and Exc-4 using the GST Gene Fusion System Followed by Affinity Chromatography

Glutathione S-Transferase (GST) Gene fusion system (originally from AMRAD-Pharmacia) was used for the expression and purification of fusion proteins in *E.coli* bacteria. Glycerol stocks of *E.coli* bacteria strain, BL21 (DE3) transformed with the pGEX-6P-1vector (GE Healthcare) containing either the open reading frame for CLIC3-

wt, CLIC4-wt, EXC-4, or CLIC3-Cys22,25A and CLIC3-Cys22A mutants, was inoculated in 100mL LB medium containing 100µg/mL Carbenicillin and left to grow on a shaker of 180 rpm, at 37°C for 16 hours . Then the grown bacterial cells were transferred into 1.5L of 2xYT medium also containing Carbenicillin 100µg/mL in order to scale up the culture of cells that were left to grow on a shaker of 180 rpm, at 37°C for 2.5 hours or until an OD of 600 was achieved (OD=0.6 at 600nm). Cells then were induced with 1mM IPTG and returned to incubation for another 2 hours at 30°C with 180 rpm shaking.

The induced cells were harvested and pelleted by centrifugation in a CR22GIII High Speed Refrigerated Centrifuge (Hitachi) using an R13A at 8000 rpm for 15 minutes at 4°C, the supernatant was poured off and cells pellet was resuspended in 15mLs of PBS buffer (0.01M phosphate buffer, 0.0027M KCl, 0.14M NaCl, 0.05% Tween containing 0.5mM TCEP, pH 7.4).

The resuspended *E.coli* cells were subsequently homogenized by ultrasonic method (as described in details mentioned in Chapter 2, Section 2.5.1.4). The supernatant containing soluble proteins were collected and filtered through a 0.45 µm filter to remove any remaining cell debris and subjected to further purification.

Then purification of the supernatant fraction was prepared first by equilibrating 3mL of glutathione-sepharose 4B resin (GE Healthcare) with ~300mL of PBS buffer (containing 0.5mM TCEP); and second by incubating the supernatant with the resin beads for 1 hour at 4°C while rocking. Then the mixture of supernatant and resin was poured into disposable chromatography column with a frit (Biorad) and washed with ice cold 300mL of PBS buffer (containing 0.5mM TCEP), then column containing resin and GST-tagged proteins were further equilibrated with 50mL of thrombin cleavage buffer (150mM NaCl, 2.5mM CaCl2, 1mM sodium azide, 0.5mM DTT or 0.5mM TCEP and 20mM Tris-HCl, pH 8). Cleavage of the CLIC3 protein from the GST that was bound to the resin beads

was then achieved by incubating them with 30 NIH units per 1L of bovine plasma thrombin and left overnight at 4°C.The cleaved CLIC3 was then eluted as 5 x 2.5 mL fractions with PBS buffer (10mM phosphate buffer, 2.7mM KCl, 140mM NaCl, pH 7.4) containing 0.05% Tween20, 0.5mM TCEP.

In order to regenerate the chromatography column, the GST-tag was removed from the sepharose resin. This was achieved by washing the column with 50mL of glutathione wash buffer (10mM L-glutathione reduced, 50mM Tris-Cl, pH 8.0) to enable the GST-tag to be eluted off the column. The column was then washed extensively and then equilibrated with 200mL PBS buffer (pH 7.4) and stored short term at 4°C or stored in PBS with 1mM sodium azide for longer term storage.

2.5.2. 21 Preparation of recombinant CLIC4, EXC-4, CLIC3 Cys-22,25A and CLIC3-Cys22A

Procedures for the expression, purification and quantification of recombinant CLIC4-wt, EXC-4, CLIC3-Cys-22,25A and CLIC3-Cys22A mutants are similar to those described above in Section 2.5.1.7.

2.5.2.22 Second Stage in Protein Purification - Size Exclusion Chromatography

In order to achieve high purity reduced recombinant CLIC proteins, Size Exclusion Chromatography (SEC) was used using a HiPrep^{TM} 16/60 Sephacryl$^{®}$ S-100 column (GE Healthcare). This second purification step, following the first round of affinity chromatography, was used to remove the thrombin enzyme and remnant contaminants following cleavage of the GST or His tag from the respective CLIC protein. The fractions of interest collected from the Nickel affinity chromatography column (Ni-NTA) or the

Glutathione affinity column, were pooled and injected onto a HiPrep™ 16/60 Sephacryl® S-100 column at +4ºC and the resultant largest single peak collected, that corresponded to the monomeric CLIC protein.

SEC by gel filtration is based on the fractionation of proteins by their size and/or their molecular weight. Different molecules in a solution are separated as the sample flows through the porous inert matrices, characterised by an exclusion limit. The exclusion limit on the Sephacryl S-100 column is 1 kDa to 100 kDa, meaning molecules in this range are able to enter the pores in the beads. The smallest molecules are able to enter more pores and are retained in the beads for a longer time, resulting in them being eluted last, while molecules of a greater size remain in the mobile phase and are eluted first. Therefore, a massive advantage of using SEC is its ability to separate different oligomeric forms of the protein resulting in the collection of either monomeric (27 kDa) or dimeric (46 kDa) or other multimeric forms of the CLIC1 proteins.

Size exclusion chromatography was performed using the ÄKTA prime plus system (GE Healthcare). Prior to start of the protein purification, the system was washed and pre-equilibrated with degassed filtered milliQ H2O followed by Column Sizing Buffer pH 7.5 (100 mM KCL, 20 mM Hepes, 1 mM sodium azide, 1 mM DTT or 0.5 mM TCEP). HiPrep™ 16/60 Sephacryl® S-100 column was attached to the ÄKTA prime plus system through the inlet and outlet tubing and manually washed with 1 column volume (CV) of Column Sizing Buffer. A maximum column pressure of 0.15 MPa and flow rate of 0.3 ml/min was fixed for all purification methods. After pre-equilibrating the system and column with Column Sizing Buffer, the specific CLIC protein, thrombin solution was applied to a 15 ml sample loop and a pre-programmed method that transferred the sample to the column at a flow rate of 0.25 ml/min was commenced. Eluted fractions were collected in volumes of 2 ml. The purity of the eluted CLIC protein was then determined

by Western Blot analysis and HEDS enzyme assay was performed to check for functionality. At the end of the purification run, the column was rinsed with at least 2 CV of Column Sizing Buffer at a flow rate of 0.3 ml/min to remove any impurities and was then disconnected from the ÄKTA prime plus system. Finally, the system was rinsed 2-3 times with degassed filtered milliQ H_2O and then stored in 20% ethanol to prevent microbial contamination.

2.5.2. 23 Preparation of Recombinant His tagged HcTrx-5 Protein

The cDNA clone encoding the protein HcTrx-5 isolated from *Haemonchus contortus* (53) was kindly provided by Associate Professor Mary Davey, from the University of Technology, Sydney, Australia. The purification of recombinant protein HcTrx-5, was done as described in the following sections.

2.5.2. 24 Small Scale Cultures

The glycerol stock containing pTrcHisB vector (Invitrogen, Australia) to express HcTrx-5-wt protein was used to inoculate individual sterile conical flasks containing 20 ml of 2xYT media and 20 µl of carbenicillin antibiotic with stock concentration of 30 mg/ml (Sigma Aldrich). These were left to grow overnight in a shaking incubator set at 200 rpm, at 37°C.

2.5.2.25 Large Scale Culture and Induction of HcTrx-5 Protein Expression

The entire volume of the small-scale cultures as described above were subsequently added to individual large flasks that contained 350 ml of 2xYT media and 30 mg/ml carbenicillin antibiotic with stock concentration of 30 mg/ml (Sigma Aldrich). After that, the flasks were left to grow in a shaking incubator at 200 rpm at 37°C for 1.5 hours. Subsequently, once the bacterial growth achieved an optical density (OD) at 600 nm value

between 0.6-0.8, 1 mM of IPTG (Isopropylthiogalactoside) was added to the media to induce the expression of the His-tagged proteins. Finally, the bacterial cultures were incubated at 20°C for a further 16 hours with shaking at 200rpm.

2.5.2. 26 Harvesting and lysing HcTrx-5-transformed E.coli Cells

The induced cells were subsequently harvested by centrifugation using High Speed Refrigerated Centrifuge (Hitachi CR22GIII) fitted with a rotor R13A rotor at 8000 rpm for 30 minutes at 4°C. the resulting bacterial pellets were collected and pooled in a 50ml falcon tube, The pellet was either stored at - 80°C for later use and the resultant pellets were scraped resuspended in the Native IMAC lysis buffer containing 1mg/mL lysozymes and 10% N-lauryl sarcosine from (Bio-Rad). Resuspended *E.coli* cells were incubated on ice for 1 hour before sonication on ice, with 10 seconds pulses at 60% output. Cell lysate was then centrifuged at 12000g for 20 minutes at 4°C. The supernatant was kept on ice and prepared to the purification of protein. Purification and Quantification of HcTrx-5-wt Protein His-tagged HcTrx-5 protein was purified using the Native IMAC purification Kit and the Profinia purification system (Bio-Rad), following manufacturer's instructions. Then the purified HcTrx-5 protein was quantified as described previously in Chapter 2, section 2.6. Also, for further confirmation of protein purity, it was run on SDS-PAGE as outlined in section 2.6.3. (54).

2. 6 Protein assays and analysis

2.6. 5 Protein quantification

Protein concentration of recombinant purified proteins was determined using a Bicinchoninic acid assay (BCA) protein estimation kit as per the manufacturer's instructions (Bio-Rad). The Bicinchoninic acid assay is a colorimetric method for the detection and quantification of total protein concentration in a solution. This assay is

based on a shift in the absorbance maximum when Coomassie® Brilliant Blue G-250 dye associates with proteins and the protein concentration quantified using the Lambert-Beer's Law. The Bradford reagent was mixed with the protein sample in a volumetric ratio of 1:1, incubated for 5 minutes at room temperature (RT) and absorbance was determined at 595 nm. Measurement readings were carried out in triplicates. A calibration curve was established each time a protein assay was performed with bovine serum albumin (BSA) dilutions of known concentrations of 0 – 2 mg/ml. Using the standard curve, the protein concentration of each sample was determined according to its absorbance by interpolation (55).

2.6. 6 Polyacrylamide Gel Electrophoresis (SDS-PAGE): Preparing and running a polyacrylamide gel

Polyacrylamide gels were prepared using a Biorad gel apparatus as per manufacturer's instructions. The gel plates, plate holders and combs were rinsed thoroughly and wiped with 80% Ethanol to remove gel residues. According to the manufacturer's instructions, 12.5% SDS-polyacrylamide gels were prepared using 10 ml of Next Gel®12.5% Acrylamide solution (Amresco) and polymerisation was initiated using 60 µl of ammonium persulphate solution (100 mg/ml in H2O) and 6 µl of NNNN'-tetramethylethylenediamine (TEMED) prior to pouring the gels. The comb was gently placed in the stacking gel. The gels were allowed to set at RT for approximately 1-2 hours and then submerged in running buffer in the Cell running tank. The running buffer was prepared by diluting 50 mL of 20x Next Gel®Running buffer (Amresco) in 950 mL of milli-Q H2O.

2.6. 7 Running SDS-PAGE gels

Sodium Dodecyl Sulfate -Polyacrylamide (SDS-PAGE) gels were run in Biorad Mini Transfer-Blot®Cell to analyse the protein samples. For SDS-PAGE electrophoresis, cell lysates and protein samples were prepared by a 1:1 dilution with 4x Laemmli buffer pH 6.8 (20% glycerol (v/v), 2% SDS, 0.02% bromophenol blue, 2mM DDT and 90 mM Tris-HCl). The samples were boiled at 95°C for 10 minutes prior to being loaded onto the gels. The gels were run at 150V for approximately 1-2 hour. The Precision Plus ProteinTM Unstained Standard (Bio-Rad) was used to estimate the apparent MW on the gel. On completion of a successful electrophoresis run, gels were stained for approximately 1 hour with Coomassie Staining (0.2% Coomassie G-250, 10% acetic acid and 40% methanol) and destained overnight using Destaining buffer (10% acetic acid and 50% methanol). Gels and protein bands were then imaged with Epson perfection 3490 flatbed scanner.

2.6. 8 Western Blotting

Western Blotting was used to further analyse the purity of CLIC1-wt proteins after SEC, protein fractions were analysed using NuPAGE®Novex Bis-tris Mini Gels according to the manufacturer's instruction (Invitrogen). The samples were prepared as mentioned above and were loaded onto a 12 well NuPAGE® Novex® 10% BisTris gel (Invitrogen). The voltage was set to a constant 200 V and the gel was run for approximately 30 minutes in 1x NuPAGE®MES SDS Running Buffer pH 7.3 (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA). After electrophoresis, samples were transferred onto a polyvinylidene fluoride membrane (PVDF) (iBlot®2 Transfer Stacks, PVDF, regular size kit) for 7 minutes at 20 V using an iBlot2 (Life Technologies) according to the manufacturer's instruction. Afterwards, PVDF membrane was blocked with 2% (w/v)

BSA in PBST pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, and 0.1% Tween 20) and gently shaken overnight at 4°C. After washing 3x for 10 minutes with PBST, the membranes were incubated for 1 hour with primary antibodies at the corresponding concentrations at 4˚C. After incubation, the membrane was washed 3x for 10 minutes with PBST, followed incubation with the corresponding conjugated secondary antibody, the anti-mouse IgG-Alkaline phosphatase (Santa Cruz Technologies, Australia) for an additional 1 hour. Excess secondary antibody was removed by washing the membrane 3x for 10 minutes with PBST. Detection was performed using the protocol for the SIGMAFASTTM BCIP®/NBT detection kit provided by Sigma Aldrich (Australia).

2. 7 HEDS Enzyme Assay

HEDS enzyme assay was performed according to the method described in Al Khamici *et al*. (2015) to evaluate the functional activity of the purified CLIC1wt protein (56). In this project, HEDS enzyme assay was performed for both WT CLIC proteins and the mutant forms of CLIC proteins. In this assay, the reduced monomeric CLIC proteins were used as test proteins while HcTrx-5 (IS5) protein, which was obtained from parasitic nematode, *Haemonchus contortus* and is known as a thioredoxin-like protein was also purified as previously described (57) and used as positive control. Each enzymatic assay was performed in triplicate in a 96-well plate containing the same final concentration of 10 µM for each CLIC proteins were added to a potassium phosphate buffer (5 mM pH 7) that contained final concentrations of 1 mM EDTA, 250 µM NADPH, 50 nM Glutathione Reductase and 1 mM HEDS, the mixture was incubated at 37°C for 5 minutes. Enzymatic reaction was initiated by the addition of 1 mM GSH to make up a final volume of 200 µl. Consumption of NADPH was monitored at A340 nm using a BioTek Power Wave TM microplate spectrophotometer. All kinetic data analysis was performed using Microsoft

Excel 2010. and equivalent amount of IS5 was used as a positive control in the HEDS enzyme assay.

In order to determine the optimal reaction conditions for the HEDS assay, we tried various concentrations of the CLICs wt proteins and the mutant forms of CLIC proteins. Also, we performed the HEDS assay for the CLICs wt proteins and the mutant forms of CLIC proteins at different range of the buffer pH.

2. 8 Pre-incubating CLIC Proteins with Ion Channel Blocker Drug

Stock solutions of 560 μM of IAA-94 were freshly prepared by dissolving 0.2mg/mL of the drug in ethanol with vortexing until a homogeneous solution of the drug in ethanol was achieved. IAA-94 were further diluted to 10μM in 5mM potassium phosphate buffer (pH 7.5). 10μM final concentration of CLIC1,3 were incubated with 10μM IAA-94 for 1 hour prior to performing the HEDS enzyme assay.

2. 9 Results

2.9. 4 Preparation of Recombinant CLIC Proteins for Use in Subsequent Assays

Homologous over-expression of recombinant CLIC1-wild type (CLIC1-wt) protein was enabled by the inducible expression vector system, pET-28a, into which the CLIC1 cDNA was cloned. The pET vector system uses a strong transcriptional promoter, pT7 to induce expression of the CLIC1 coding sequence placed downstream from it. The pT7 transcriptional promoter and IPTG inducible promoter controls the expression of bacteriophage T7 RNA polymerase; this is highly selective and active that, when fully induced almost all of the cell's resources are directed towards the expression of the target gene and production of the encoded protein. Hence, the expression of the CLIC1 gene is induced by the addition of the lactose analogue Isopropyl-thio-β-D-galactopyranoside

(IPTG); use of this operon allows the expression of T7 RNA polymerase, which in turn transcribes the CLIC1 gene. CLIC1-wt over-expression and purification were based upon the methods used by Valenzuela *et al*. (1997) (58). Glycerol stocks of *Escherichia coli* BL21 (DE3) (Stratagene, USA), transformed with the CLIC1-pET-28a plasmid were purified and DNA plasmids pET28a (empty vector) and pET28a containing the cDNA sequence encoding wild-type CLIC1protein, where digested using the restriction enzymes Not1 and Nde1. Then the products were electrophoresed on agarose gel (1%). The resulting of a single band at ~725 bp. in Lane 6 (Figure 2.4) clearly indicates the presence of CLIC1 open reading frame (ORF) insert at its expected molecular weight.

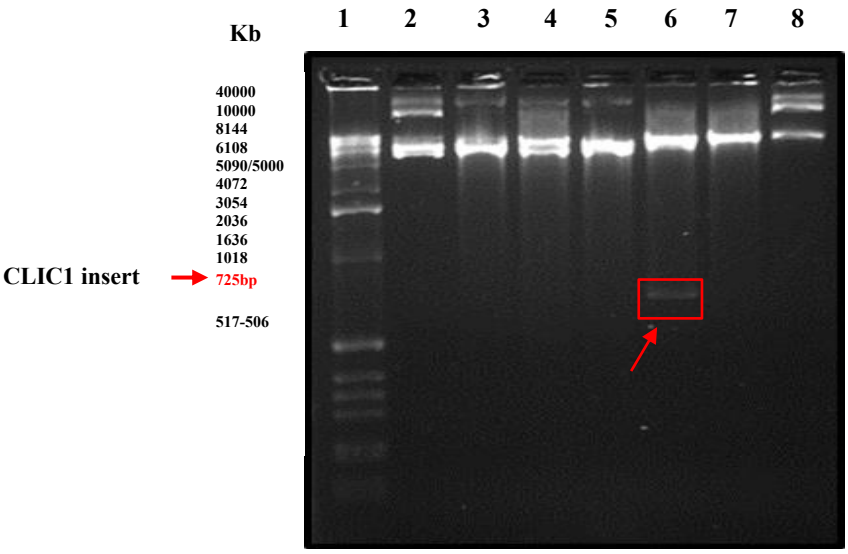

Figure 2. 4 CLIC1 cDNA Expression Construct.

Agarose gel electrophoresis analysis of CLIC1 cDNA clone (in pET28a vector) following restriction enzyme digest. Lane 1. Standard DNA Ladder 2 µl, Lane 2. Uncut pET28a vector 10 µl, Lane 3. pET28a vector cut with Nde1 10µl, Lane 4. pET28a vector cut with Not1 10µl, Lane 5. CLIC1-wt in pET28a vector cut with Nde1 10µl, Lane 6. CLIC1-wt in pET28a vector cut with Nde1/Not1 10µl, Lane 7. CLIC1-

wt in pET28a vector cut with Not1 10µl, Lane 8. CLIC1-wt in pET28a vector uncut 10µl.

Moreover, the plasmids pGEX-6P-1containing the open reading frame for the proteins CLIC3-wt, CLIC3-Cys22A and CLIC3-Cys22,25A mutants obtained from Dr Sara Zanivan (Glasgow, UK) were transformed in bacterial strain *Escherichia coli* BL21 (DE3). The homologous over-expression of recombinant CLIC3-wt protein was enabled by the inducible expression vector system, pGEX-6P-1, into which the CLIC3 cDNA was cloned. The DNA plasmid pGEX-6P-1 (empty vector) or pGEX-6P-1 containing the cDNA encoding the wild-type CLIC3 protein or its mutants CLIC3-Cys22A or CLIC3-Cys22,25A were purified and digested with the restriction enzymes at Not1 and Bam1 site. The products were electrophoresed on agarose gel (1%) and the resulting single bands in Lane 5,7,9 indicating the presence of CLIC3 ORF insert for wild-type CLIC3 protein and its mutants CLIC3-Cys22A and CLIC3-Cys22,25A as a single band at ~708 bp. (Figure 2.5).

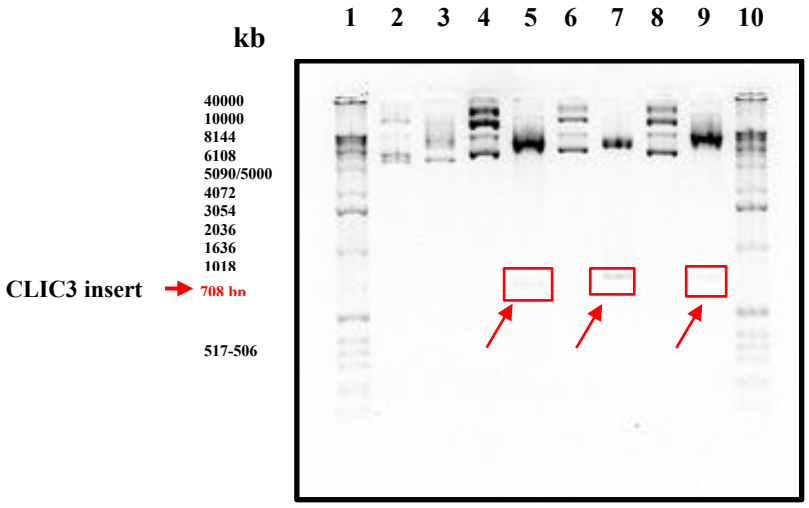

Figure 2. 5 CLIC3 cDNA Expression Construct.

Agarose gel electrophoresis analysis of CLIC3 cDNA clone (in pGEX-6P-1 vector) following restriction enzyme digest. Lane 1. Ladder 2 µl, Lane 2. Uncut pGEX-6P-1

vector 10 µl, Lane 3. pGEX-6P-1 vector cut with Not 1/Nde 1 10µl, Lane 4. CLIC3-wt in pET28a vector uncut 10µl, Lane 5. CLIC3-wt in pET28a vector cut with BamH1/Not1 10µl, Lane 6. CLIC3-Cys22A in pGEX-6P-1 vector uncut 10µl, Lane 7. CLIC3-Cys22A in pGEX-6P-1 vector cut with Not 1/BamH1 10µl, Lane 8. CLIC3- Cys22,25A in pGEX-6P-1 vector uncut 10µl, 9. CLIC3- Cys22,25A in pGEX-6P-1 vector cut with Not 1/BamH1 10µl, 10. Ladder 2 µl.

In addition, the pGEX-2T plasmids containing the open reading frame for the proteins CLIC4-wt was obtained from Prof. Paul Curmi's Group at the School of Physics, University of New South Wales, Sydney, Australia. Homologous over-expression of recombinant CLIC4-wild type protein was enabled by the inducible expression vector system, pGEX-2T, into which the CLIC4 cDNA was cloned. Also, the digestion of DNA plasmids from the wild-type CLIC4 in pGEX-2P-T, pGEX-2P-T (empty vector) with the restriction enzymes at *BamH1* and *HindIII* site. Then the products were electrophoresed on agarose gel (1%) and indicated a single band at ~759 bp. Lane 6 which referred to the CLIC4 insert (Figure 2.5).

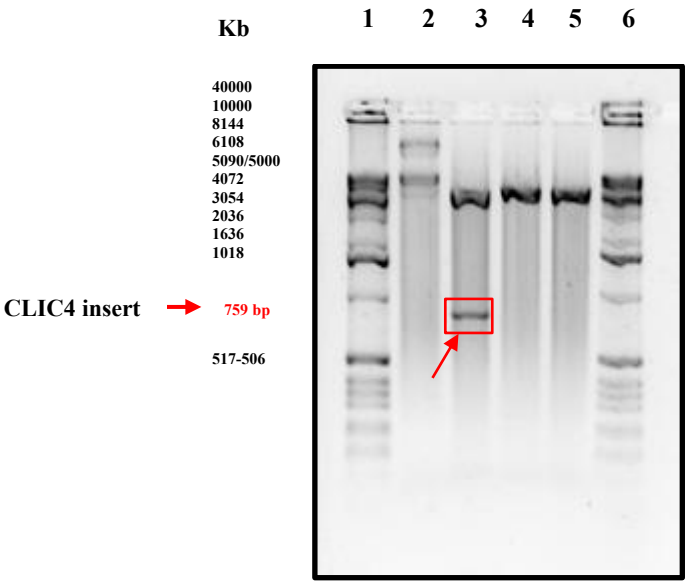

Figure 2. 6 CLIC4 cDNA Expression Construct.

Agarose gel electrophoresis analysis of CLIC4 cDNA clone (in pGEX-2T vector) following restriction enzyme digest. Lane 1. Ladder 2 µl, Lane 2. CLIC4-wt in pGEX-2T vector uncut 10 µl, Lane 3. CLIC4-wt in pGEX-2T vector cut with BamH1/ HindIII 10µl, Lane 4. CLIC4-wt in pGEX-4T vector cut with HindIII 10µl, Lane 5. CLIC4-wt in pGEX-2T vector cut with BamH1 10µl Lane, 6. Ladder 2 µl.

Based on these observations, the presence of the CLIC1, 3 and 4 cDNA inserts at their expected molecular weight as a single band confirms their correct cloning into their respective vectors and their stable transformation into the bacterial cells *Escherichia coli* BL21 (DE3).

Recombinant CLICs-wt and the mutant forms of CLICs protein were readily purified from *E. coli* cell lysates following IPTG induced expression, followed by two subsequent chromatography steps. The overexpression conditions for CLICs-WT and mutants had been previously determined and the recombinant proteins were over-expressed and purified as described in the methods Section (2.7.1 and 2.7.2). The initial purification step was performed manually by nickel-affinity chromatograph (for the His tagged proteins) and Glutathione affinity column for the GST fusion proteins, yielding a protein recovery of 10-15 mg per liter bacterial culture.

2.9. 5 Size Exclusion Chromatography Results

Purification of the CLIC proteins used a two-stage purification process, first using affinity chromatography followed by size exclusion chromatography (SEC). The various fractions from the affinity chromatography were checked by SDS-page. Thrombin was used to cleave the 6x Histidine tag from the CLIC1 protein (Figure 2.7). A representative

profile for the SEC eluted monomeric CLIC1-wt protein is shown in Figure 2.7, detected at an absorbance of 280 nm. Fractions 23 to 30 were pooled, and protein quantification and SDS-PAGE analysis performed.

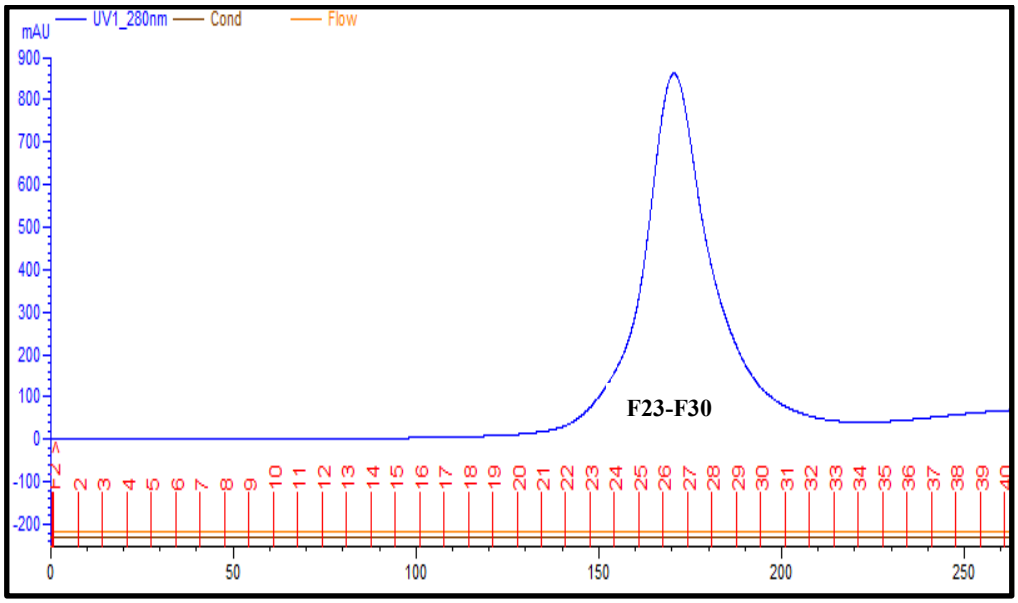

Figure 2. 7 Eluted fractions of the CLIC1-wt protein from Size Exclusion Chromatography Column. *The large peak area of the fractions (F23-30) represent total of 21ml of eluted CLIC1-wt protein that was aliquoted and stored at -80°C.*

2.9. 6 SDS-PAGE and Western Blot analysis of samples collected following Affinity and SEC Chromatography

In order to determine the purity and confirm the expected molecular weight of all CLIC proteins, SDS-page electrophoresis was conducted following purification of CLIC proteins through the affinity chromatography and size exclusion chromatography columns. Examples from CLIC1, 3 and 4 are outlined below.

As seen in (Figure 2.8), Lane 6 represents the flow through off the Ni-NTA column and indicates that considerable amount of CLIC1-wt fusion protein is bound to the column.

The protein band in Lane 7 shows the CLIC1-wt following thrombin digestion purified by size exclusion chromatography and the band correlates to the theoretical molecular mass of approximately 27 kDa for CLIC1-wt. Lane 8 of (Figure 2.8), represented the control band of CLIC1-wt proteins previously purified by SEC, indicates that HisCLIC1-wt protein was successfully purified.

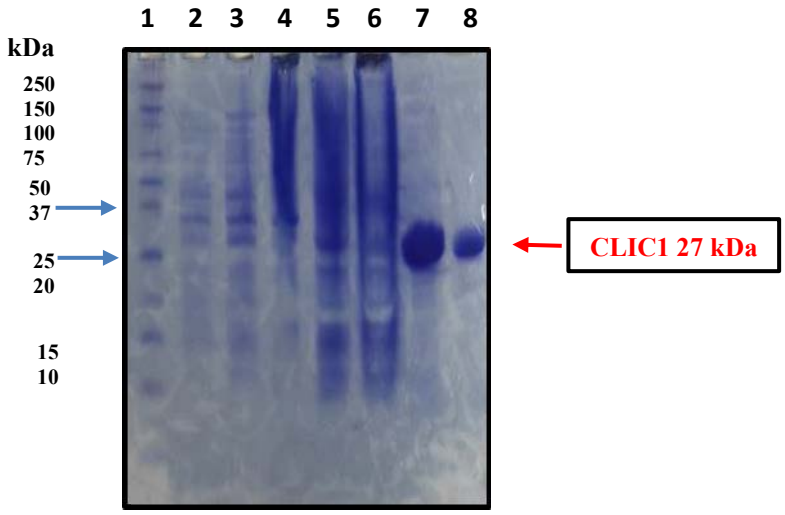

Figure 2. 8 SDS-PAGE of CLIC1-wt protein expression and purification. *Lane 1- Protein Ladder with estimated molecular weights of proteins (Bio Rad), Lane 2- sample of un-induced E-coli bacterial cells, Lane 3- sample of induced E-coli cells with 1mM IPTG, Lane 4- sample of the soluble proteins following cell lysis, Lane-5 sample of pellet, Lane 6- eluted sample from the His-tag high affinity chromatography column prior incubation with thrombin sample of supernatant, Lane 7- Purified CLIC1-wt by size exclusion chromatography and Lane 8- recombinant CLIC1-wt protein used as a control.*

The purity of the recombinant CLIC4-wt protein was assessed by SDS-PAGE (Figure 2.9). The protein band in Lane 2 and 3 of Figure 2.9 shows the CLIC4 fractions samples following thrombin digest and purification by size exclusion chromatography to remove

GST. The band correlate to the theoretical molecular mass of approximately 28.6 kDa for CLIC4-wt fractions purified by size exclusion chromatography. Purified CLIC1 protein by size exclusion chromatography used as a control in Lane 4 and 5 of Figure 2.8, indicated that CLIC4-wt protein was successfully purified. In Lane 1 of (Figure 2.9), the ladder with estimated molecular weights of proteins showed the presence of CLIC4-wt band of approximately 28.6 kDa.

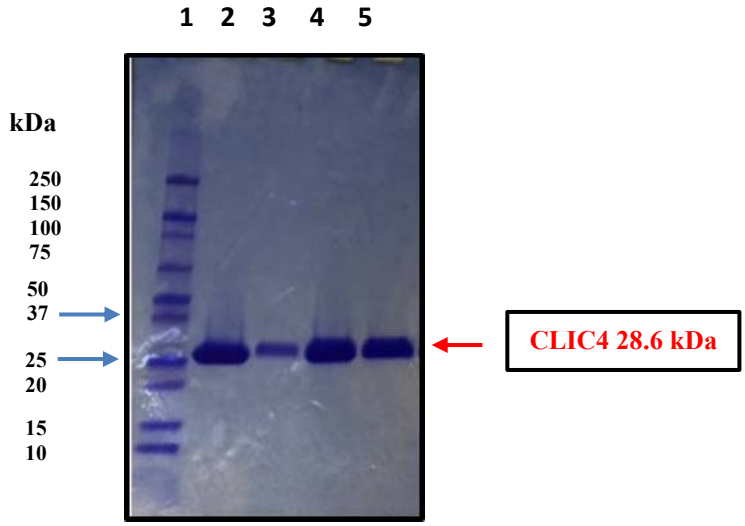

Figure 2. 9 SDS-PAGE of CLIC4-wt protein expression and purification. *Lane 1- Ladder with estimated molecular weights of proteins, Lane 2- Purified CLIC4-wt by size exclusion chromatography, Lane 3- Purified CLIC4-wt by size exclusion chromatography, Lane 4- Purified CLIC1-wt by size exclusion chromatography used as a control and Lane-5 Purified CLIC1-wt by size exclusion chromatography used as a control.*

Finally, the purity of the recombinant CLIC3-wt protein was assessed by SDS-PAGE (Figure 2.10). The protein band in Lane 2 and 3 of Figure 2.10 shows the CLIC3-wt fractions samples following thrombin digest and purification by size exclusion chromatography to remove GST. The band correlate to the theoretical molecular mass of

approximately 26.7 kDa for CLIC3-wt fractions purified by size exclusion chromatography. Purified CLIC1-wt protein by size exclusion chromatography used as a control in Lane 4 and 5 of Figure 2.10, indicated that CLIC3-wt protein was successfully purified the. In Lane 1 of (Figure 2.10), the ladder with estimated molecular weights of proteins showed the presence of CLIC3-wt band of approximately 26.7 kDa.

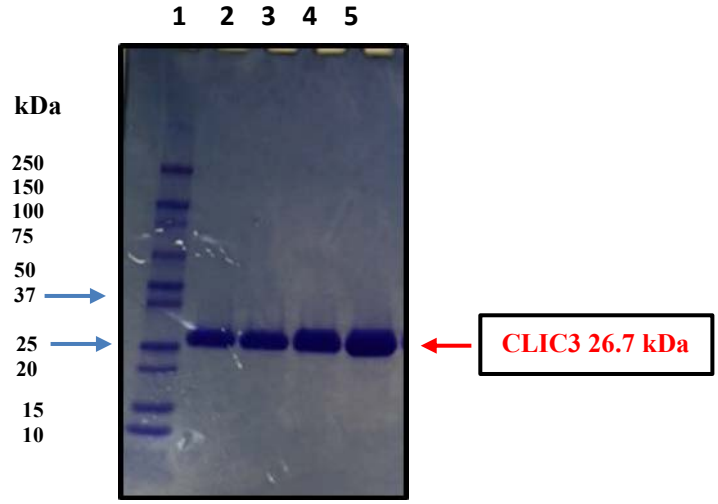

Figure 2. 10 SDS-PAGE of CLIC3-wt proteins expression and purification. *Lane 1- Ladder with estimated molecular weights of proteins, Lane 2- Purified CLIC3-wt by size exclusion chromatography, Lane 3- Purified CLIC3-wt by size exclusion chromatography, Lane 4- Purified CLIC1-wt by size exclusion chromatography used as a control and Lane-5 Purified CLIC1-wt by size exclusion chromatography used as a control.*

Based on these observations, the consistent presence of single bands for CL_ _ 1, 3 and 4 in (Figures 2.8, 2.9 and 2.10) at their expected molecular weights, strongly indicated that the CLIC proteins were successfully over-expressed and purified from *E. coli* cell lysates. Similar analyses were performed for all proteins prepared and used in this project.

To verify the identity of the eluted CLIC1-wt protein in the SEC fractions, SDS-PAGE followed by western blot analysis was conducted as described in (Sections 2.6.4), CLIC proteins concentration was then determined using the Bradford protein quantification assay kit. The CLIC1 protein profiles corresponding to the fractions 23-30 were correlated with the molecular weight (27 kDa) as shown in (Figure 2.7). Western blot analysis using anti-CLIC1 antibody resulted in a single protein band of molecular mass of approximately 27 kDa, thus confirming the presence of CLIC1 protein as seen in (Figure 2.10).

These steps resulted in purification of the CLIC proteins to near homogeneity. The eluted protein fractions from the size exclusion chromatography column were aliquoted and stored in column sizing buffer (100mM KCl, 1mM NaN3, and 20mM HEPES pH 7.5; containing 0.5mM TCEP to keep the proteins in its reduced monomeric form) at -80 C°.

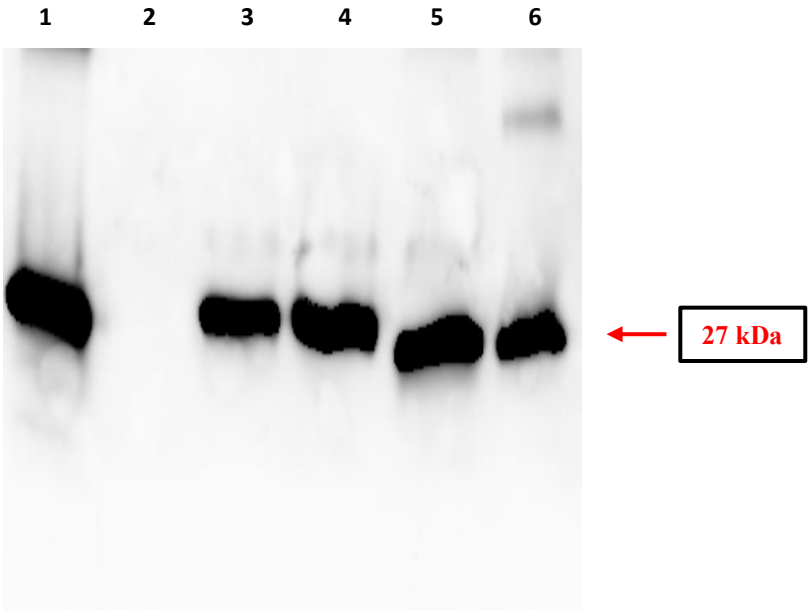

Figure 2. 11 Western blots of CLIC1-wt and mutants. *Lane 1- recombinant CLIC1-wt protein used as a control (from a previous batch), Lane 2- Crude supernatant fraction of empty vector transformed bacteria (pET28a) , Lane 3- Purified CLIC1-wt following size exclusion chromatography (Fraction 26), Lane-4 Purified CLIC1-K37A following size*

exclusion chromatography, Lane 5- Purified CLIC1-Cys24S following size exclusion chromatography, Lane-6 Purified CLIC1-R29A mutant following size exclusion chromatography.

Western blots of CLIC3-wt and CLIC4-wt were also undertaken by others in the lab group to confirm their correct identity – blot results not shown here.

2. 10 Testing for the Enzymatic Activity of CLIC Proteins in the HEDS Assay

2.10. 5 CLIC3 Protein Demonstrates Glutathione-Like Enzymatic Activity

Given the strong homology between the CLIC family members, including the conserved enzymatic active G-site motif, we hypothesised that like CLICs 1, 2 and 4, CLIC3 would also have glutathione-dependent oxidoreductase activity. In the HEDS assay, 2-hydroxyethyl disulphide (HEDS) is used as the substrate. It is a low molecular weight compound found to act as a specific and sensitive substrate for assaying glutaredoxin enzymatic activity (56).

The HEDS assay was employed to test for similar enzymatic activity by CLIC3-wt and CLIC1-wt and mutant versions of the proteins. As seen in Figure 2.12, in the presence of the positive control protein His HcTrx-5, a known glutathione-dependent oxidoreductase from *Haemonchus contortus*, the consumption of NADPH increases (resulting in a decreased A340 nm). Similar consumption of NADPH is observed when CLIC1-wt and CLIC3-wt proteins were employed. This indicates that CLIC1-wt and CLIC3-wt reduced the HEDS substrate when coupled with glutathione (GSH) and glutathione reductase (GR) in the presence of NADPH.

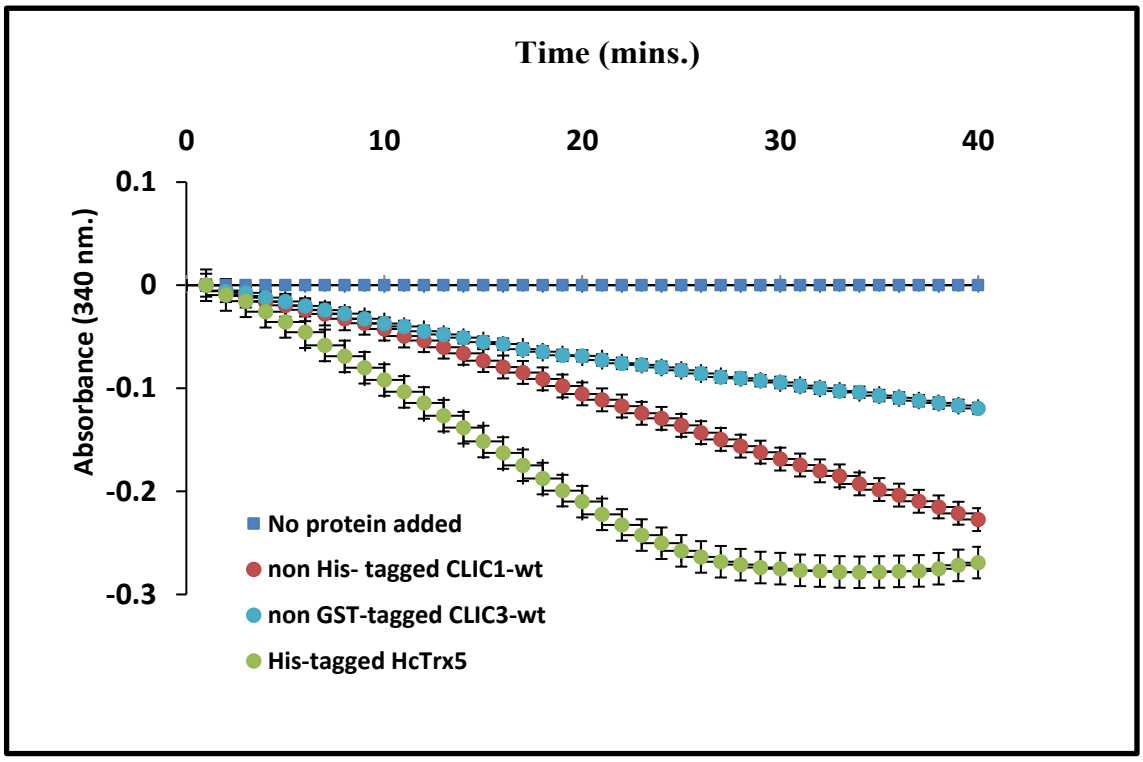

Figure 2. 12 Activity of the CLIC1-wt and CLIC3-wt proteins in the HEDS enzyme assay. *The HEDS enzyme assay was carried out using 5mM potassium phosphate with 1mM EDTA, pH 7 containing 10µM of CLIC proteins, CLIC1-wt or CLIC3-wt or His-HcTrx-5 (control protein), 250µM NADPH, 1mM HEDS and 50nM GR. The mixture was incubated at 37ºC and then the reaction was initiated by the addition of 1mM GSH and the absorbance of NADPH was monitored at A340nm. Error bars represent the standard error of at least three independent measurements.*

2.10. 6 Functional Characterisation of CLIC Proteins and CLIC1 mutants in the HEDS Assay

CLIC1 mutants: R29A, K37A, in which a single amino acid of CLIC1 was substituted to alanine (59) as shown in Figure 2.13. These two amino acids R29 and K37 are conserved in all vertebrate CLICs, and have been shown to be important for the ion channel activity of CLIC1 (60).

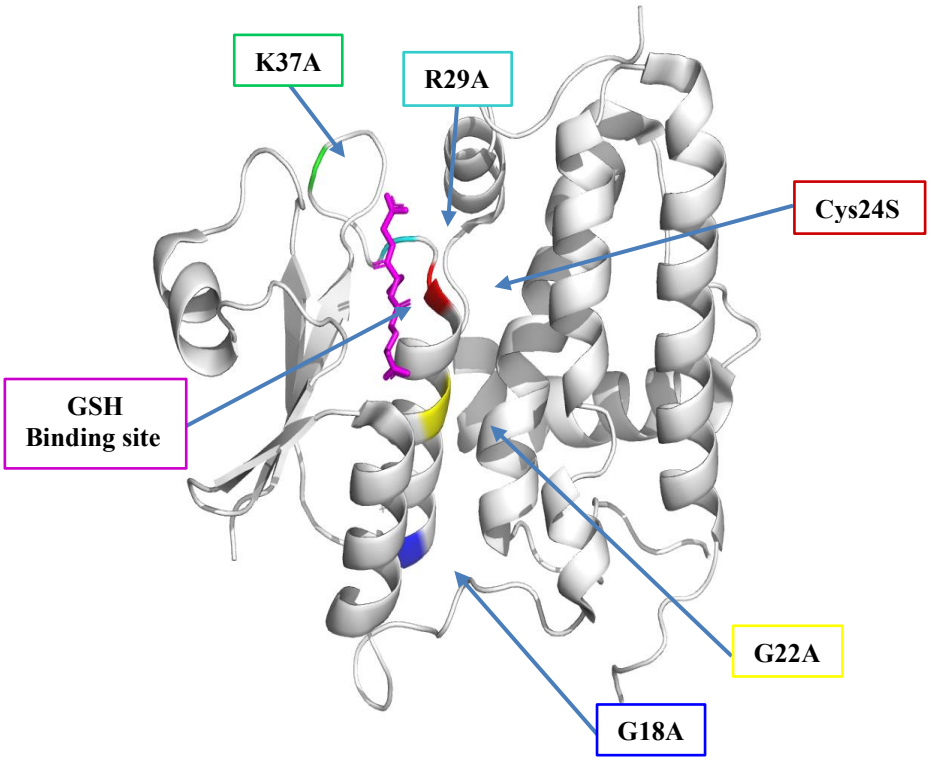

Figure 2. 7 Schematic diagram of reduced CLIC1 showing the positions of the different amino acids that were mutated to alanine. *The GSH binding site are shown in pink, lysine (K37A) in green, Arginine (R29A) in red, Cysteine (Cys24S) in cyan, Glycine (G18A) in blue and Glycine (G22A) in yellow.*

In this study, different members of the CLIC family (wild type proteins) and mutant versions were assessed in the HEDS enzyme assay. As seen in Figure 2.14, the positive control protein His-HcTrx-5, which is a worm derived enzyme found to have glutathione-dependent activity. Consumption of NADPH is observed when CLIC1-wt, CLIC3-wt, CLIC1-K37A, and CLIC1-R29A were substituted for the control protein in the HEDS assay. This indicates that all these proteins tested were able to reduce the disulfide bond of the HEDS substrate when coupled with reduced glutathione (GSH) and glutathione reductase (GR) in the presence of NADPH.

However, CLIC1-wt, CLIC3-wt, CLIC1-K37A, and CLIC1-R29A demonstrate varying levels of enzymatic activity in the HEDS enzyme assay. CLIC1 appears to have the highest oxidoreductase activity, followed by CLIC3-wt (fig 2.12). The mutants CLIC1-R29A and K37A previously shown to be important for the ion channel activity of CLIC1 (60), also demonstrate glutaredoxin-like enzymatic activity, albeit reduced, compared to wild type CLIC1-wt and CLIC3-wt protein. Furthermore, the CLIC-like protein Exc-4 showed an apparent lower enzymatic activity in the HEDS enzyme assay compared to CLIC1-wt and CLIC3-wt.

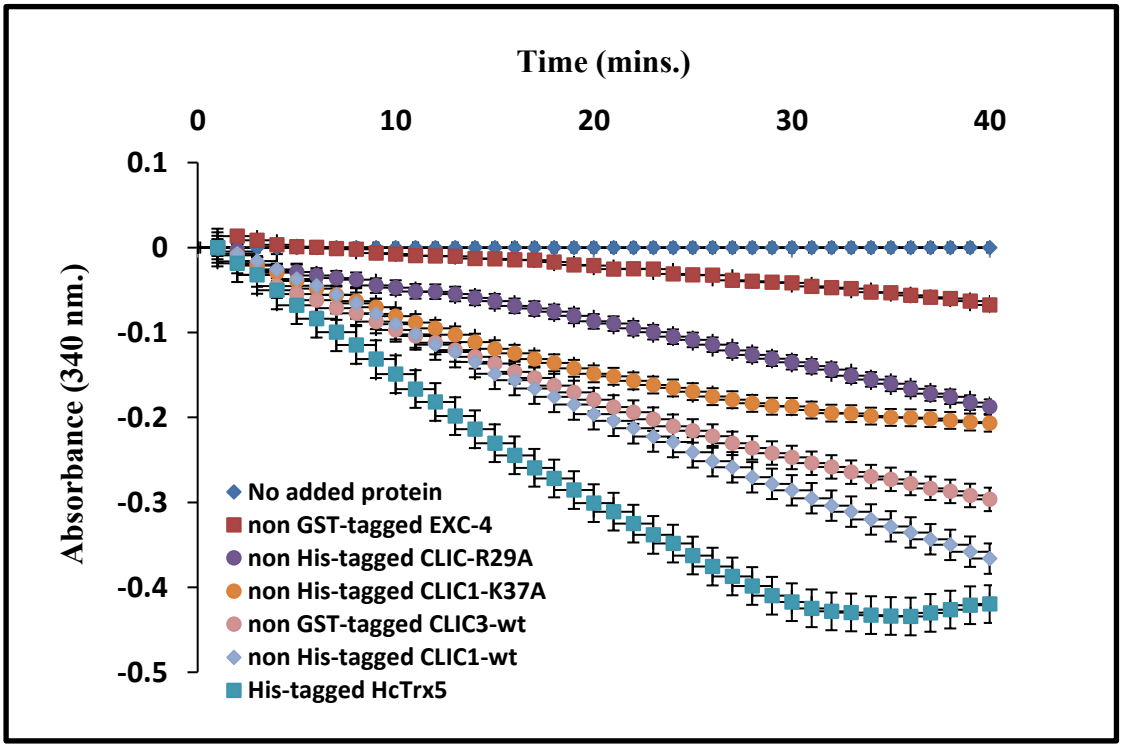

Figure 2. 8 Activity of the CLIC1-wt, CLIC3-wt, CLIC1-K37A, CLIC1-R29A proteins and the CLIC like protein Exc-4 in the HEDS enzyme assay. *The HEDS enzyme assay was carried out using 5mM potassium phosphate with 1mM EDTA, pH 7 containing 10µM of CLIC proteins (CLIC1-wt, CLIC3-wt, CLIC1-K37A, CLIC1-R29A proteins and the CLIC like protein Exc-4) or His-HcTrx-5 (control protein), 250µM NADPH, 1mM HEDS and 50nM GR. The mixture was incubated at 37ºC and then the reaction was*

initiated by the addition of 1mM GSH and the absorbance of NADPH was monitored at A340nm. Error bars represent the standard error of at least three independent measurements.

2.10. 7 Investigation of the critical Cysteine residues involved in the enzymatic activity of both CLIC1 and CLIC3

In CLIC1, Cys24 represents the monothiol redox active residue within the enzyme active site. The conserved Cys24 is essential for CLIC1 oxidoreductase activity as seen in Figure 2.15. In order to confirm Cys24 is the key active cysteine residue involved in CLIC1 oxidoreductase activity, a mutant version of CLIC1 was assayed, with Cys24 mutated to serine (C24) and tested in the HEDS assay. Cys24S mutant of CLIC1 was found to have no enzymatic activity in the HEDS assay. Furthermore, we have also tested the mutant version of CLIC1 with Cys24 mutated to alanine (C24), which was also found to have no enzymatic activity in the HEDS assay (data not shown). In CLIC3; Cys22 and Cys25 represent the dithiol redox active residues within the enzyme active site as shown in Figure 2.15.

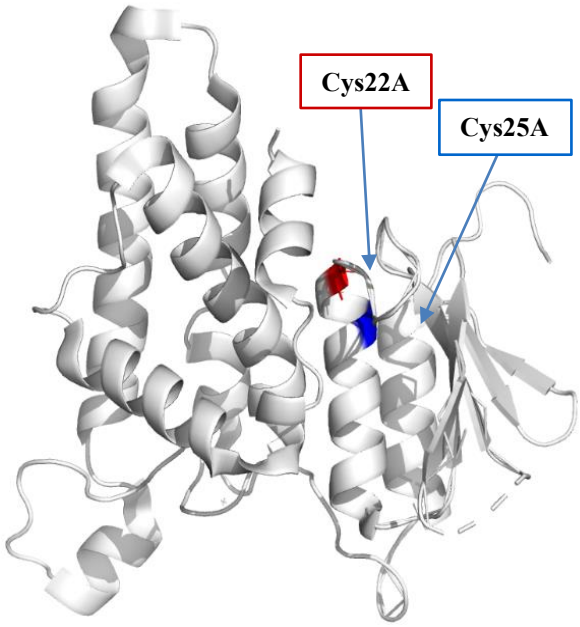

Figure 2. 9 Schematic diagram of reduced CLIC3 showing the positions of the dithiol residue (Cys22 and Cys25) that were mutated to alanine. *The Cysteine (Cys22A) are shown in red, Cysteine (Cys25A) in blue.*

The mutant versions of CLIC3 were assayed, with the single Cys22 mutated to alanine (C22A) and tested in the HEDS assay. The Cys22A mutant of CLIC3 showed significantly reduced enzymatic activity, however there remained some delayed, residual activity (Figure 2.16).

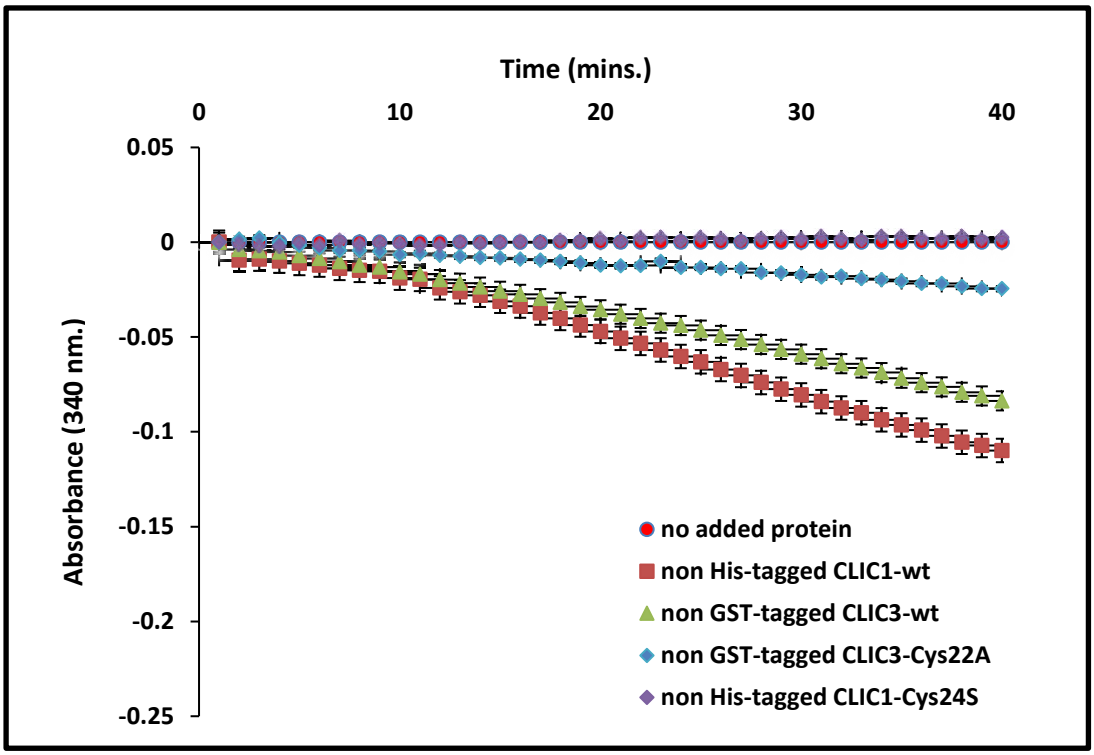

Figure 2. 10 Comparison of the oxidoreductase activity of CLIC1 and CLIC3 (WT) monomer and CLIC1 and CLIC 3, Cys mutants. *A reaction mixture of 5mM potassium phosphate (pH 7) with 1mM EDTA buffer containing 250µM NADPH, 50nM GR, 1mM HEDS and 10µM CLIC1,3 (WT) monomer, CLIC1-C24S and CLIC3-C22A that was incubated for 5 mins at 37ºC. The reaction was initiated with the addition of 1mM GSH and the absorbance of NADPH was monitored at A340nm. Error bars represent the standard error of at least three experimental repeats.*

CLIC3 contains a dithiol motif in its predicted active site. In order to confirm that both Cys22 and Cys25 are the key active cysteine residues involved in CLIC3 oxidoreductase activity, the double mutant version of CLIC3 was assayed, with both Cys22 and Cys25 mutated to alanine (C22,25A) and tested in the HEDS assay. The Cys22,25A mutant of

CLIC3 was found to have virtually no enzymatic activity in the HEDS assay (Figure 2.17).

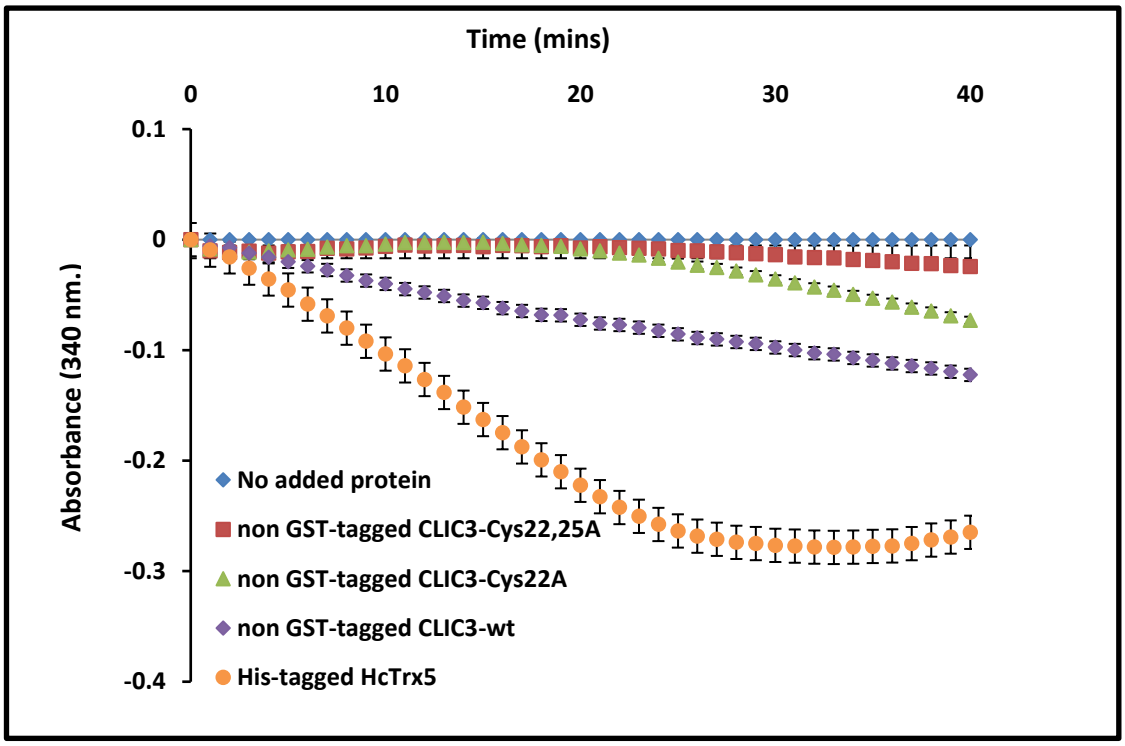

Figure 2. 11 Comparison of the oxidoreductase activity of CLIC3-wt monomer, CLIC3-Cys22A and CLIC3-Cys22,25A mutants. *A reaction mixture of 5mM potassium phosphate (pH 7) with 1mM EDTA buffer containing 250µM NADPH, 50nM GR, 1mM HEDS and 10µM CLIC3- wt monomer, CLIC3-C22A and CLIC3-Cys 22,25A that was incubated for 5 mins at 37ºC. The reaction was initiated with the addition of 1mM GSH and the absorbance of NADPH was monitored at A340nm. Error bars represent the standard error of at least three experimental repeats.*

2.10. 8 Inhibition of CLIC1 and CLIC3 Enzymatic Activity by Chloride Ion Channel Blocker Drugs

IAA-94, A9C and DIDS are known chloride ion channel blockers. Electrophysiological studies have shown that both IAA-94 and A9C block CLIC1 ion channel activity in cells (61). *In vitro* studies further confirm that IAA-94 inhibits CLIC channels produced by adding recombinant soluble CLIC1 to artificial bilayers (37, 62, 63). To investigate the effect of IAA-94 on the enzymatic activity of CLIC1-wt and CLIC3-wt, the CLIC proteins were pre-incubated with IAA-94 for an approximately 1 hour. As seen in Figures 2.18 and 2.19 below, IAA-94 completely blocked the enzymatic activity of CLIC1-wt and CLIC3-wt in the HEDS enzyme assay.

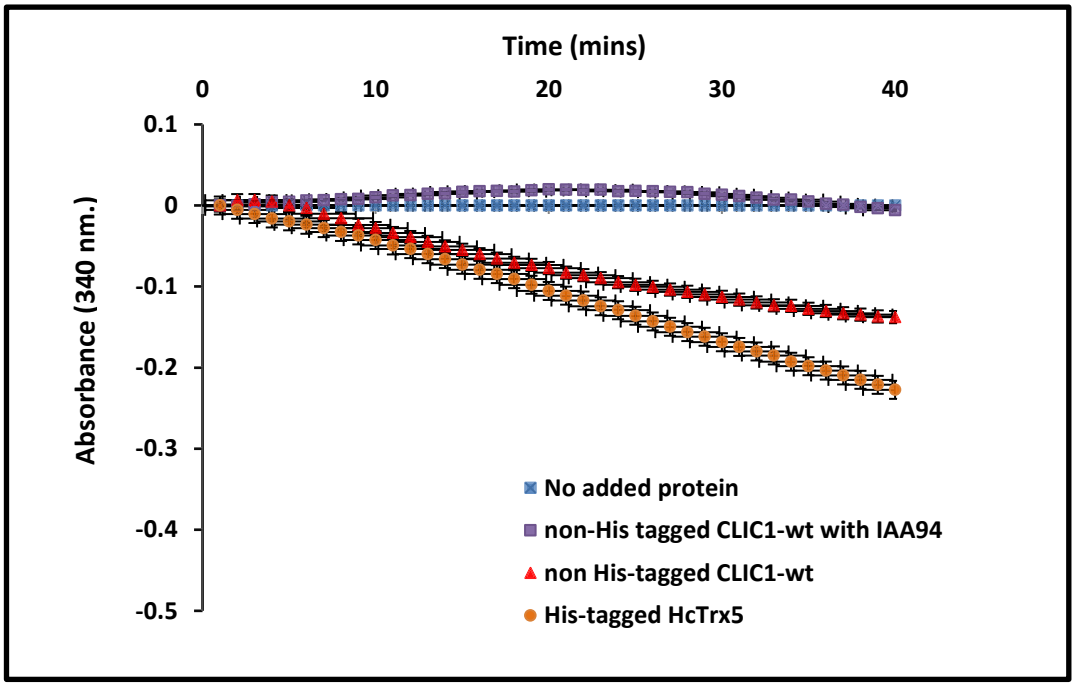

Figure 2. 12 Effect of chloride ion channel inhibitor drugs on the enzymatic activity of CLIC1 in the HEDS enzyme assay. *5mM potassium phosphate buffer (pH 7) with 1mM EDTA containing 250μM NADPH, 1mM HEDS, 50nM GR and 10μM of CLIC1 reduced*

(WT) or HcTrx-5 protein pre- incubated with 10µM IAA-94 for ~1hour prior use of the protein in the assay. The reaction mixture was incubated for 5 mins at 37ºC and then was initiated by the addition of 1mM GSH. The consumption of NADPH was monitored at A340nm post addition of 1mM GSH. Error bars shown represent the standard error of at least three experimental measurements.

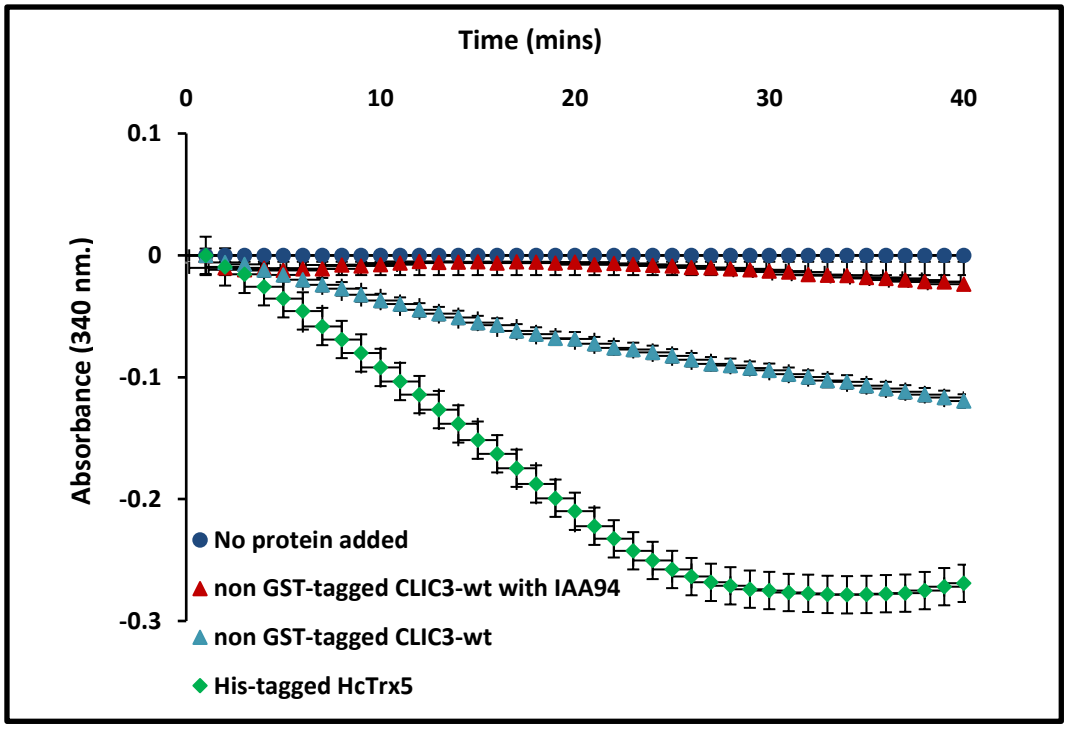

Figure 2. 13 Effect of chloride ion channel inhibitor drugs on the enzymatic activity of CLIC3 in the HEDS enzyme assay. 5mM potassium phosphate buffer (pH 7) with 1mM EDTA containing 250µM NADPH, 1mM HEDS, 50nM GR and 10µM of CLIC3 reduced (WT) or HcTrx-5 protein pre- incubated with 10µM IAA-94 for ~1-hour prior use of the protein in the assay. The reaction mixture was incubated for 5 mins at 37ºC and then was initiated by the addition of 1mM GSH. The consumption of NADPH was monitored at A340nm post addition of 1mM GSH. Error bars shown represent the standard error of at least three experimental measurements.

2. 11 Discussion

2.11. 3 Soluble CLIC Proteins Demonstrate Oxidoreductase Enzymatic Activity

CLIC proteins were initially described as chloride channels, however their functions have been extended to show that they can also act as molecular scaffolds (47) and as enzymes (49). A previous study by our group at UTS (48) has shown that the enzymatic activity of the CLIC proteins was a GSH-dependent oxidoreductase activity, which was distinct to the dithiol glutaredoxins. Three CLIC proteins were previously studied, with CLIC2-wt demonstrating the lowest level of enzymatic activity in the HEDS assay, compared to either CLIC1 or CLIC4. The apparent lower activity of CLIC2 compared to CLIC1 and CLIC4 may be related to the variation in the active site between these proteins, with CLIC2 containing the dithiol motif (CPFC), whilst both CLIC1 and CLIC4 contain the monothiol motif (CPFS). However, further studies are needed in order to establish a distinct dithiol catalytic mechanism by CLIC2 compared to the monothiol members CLIC1 and CLIC4 (48) and to ascertain optimal reaction conditions for each of the CLIC members.

In this chapter, I have demonstrated for the first time that soluble recombinant CLIC3-wt functions as an oxidoreductase enzyme (Figure 2.12) in a similar manner to other CLIC protein family members (CLIC1, CLIC2 and CLIC4) using the HEDS enzyme assay (48). In this study, we used the protein His-tagged *HcTrx-5* isolated from *Haemonchus contortus* as a positive control in our HEDS assay. The His-tagged *HcTrx-5* showed a distinct activity when it compared with our non-His or non-GST tagged CLICs proteins. Another member of our group has shown that this difference, increased activity in the HEDS assay, was largely due to the presence of the 6 x Histidine tag on the HcTrx-5

protein (*Ms Saba Moghaddasi, MSc Thesis, 2018 UTS*), hence why all experiments performed in this thesis have had all extraneous tags removed.

The HEDS enzyme assay system is considered a characteristic assay for the glutaredoxin proteins which act by deglutathionylating the mixed disulphide between glutathione (GSH) and the beta-mercaptoethanol region of the HEDS reagent. Grxs reduce the disulphide bonds of the HEDS causing the Grxs to be oxidised. The oxidised Grxs are reduced back again by GSH which will in turn be oxidised (GSSG). GSSG is then reduced again by accepting electrons from NADPH via glutathione reductase (GR) as shown in (Figure 2.20) below.

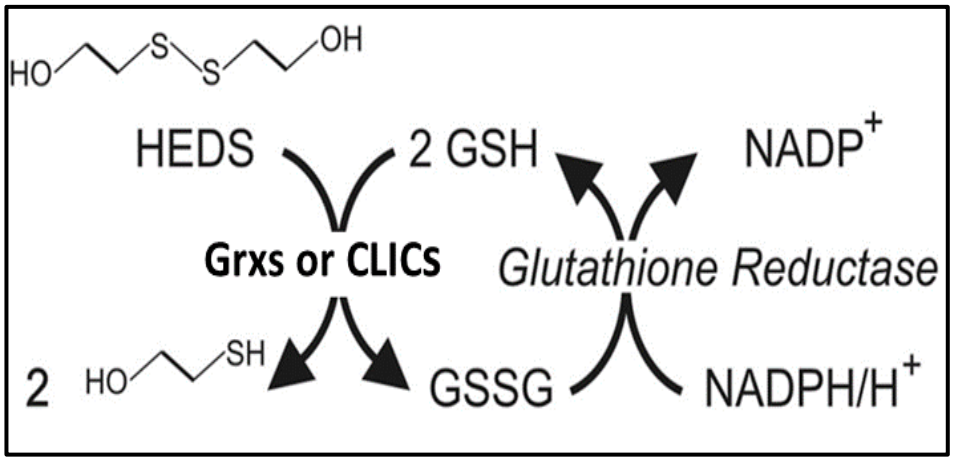

Figure 2. 14 Glutaredoxin activity in the HEDS enzyme assay. Grxs reduce the disulfide bonds of the HEDS causing the Grxs to be oxidised. *The oxidised Grxs will be reduced again by GSH which will be left oxidised (GSSG) which is returned to its reduced form again by accepting electrons from NADPH via glutathione reductase (GR). Modified Figure was taken from* **(64)**.

Like the thioredoxins and glutaredoxins, the CLIC N-terminal domain has an active cysteine that is capable of forming mixed disulfides. Moreover, the C-terminal substrate binding region of the CLICs appears large enough to accommodate peptide substrates

raising the possibility that a redox-regulated protein interaction may form part of the channel forming process (65). Reactions catalysed by glutaredoxin-like enzymes are also dependent upon the redox context of the environment. In the strongly reducing environment of the cytosol, high glutathione concentrations (0.5-10 mM) can compromise protein activity by glutathionylation, and CLICs may de-glutathionylate these cysteine residues to restore protein activity (48). However, outside the cell, where glutathione concentrations are much lower (μM range), CLICs would be less likely to function as de-glutathionylating enzymes.

Chloride Intracellular Channel Protein 3 (CLIC3) was one of the most significantly upregulated components of the iCAF proteome. Knockdown of CLIC3 in iCAFs reduced the ability of these cells to remodel the ECM and to support tumour cell invasion (49). CLIC3's association with tumour aggressiveness is likely attributable to its ability to control the recycling of lysosomally-targeted α5β1 integrin. Our collaborates at the Beatson institute for cancer research/ Glasgow university/ UK, found that there is one abundant secreted protein whose levels increased in parallel with CLIC3; this was transglutaminase 2 (TGM2) (49). Given our group's new discovery describing CLIC members thioredoxin activity (48), and studies indicating that thioredoxin activates TGM2 (66), it was proposed that CLIC3 might be able to reduce the disulphide bonds of the enzyme TGM2 to influence its activity.

Our collaborates at the Beatson institute demonstrated that CLIC3 is a secreted protein which increases both the stiffness of the ECM and the amount of fibrillar collagen in organotypic plugs. These changes in the tumour microenvironment are associated with increased tumour cell migration and invasion. Transglutaminases are cross-linking enzymes and are thought to play a role in ECM stiffness and cancer progression. TGM2

physically associates with CLIC3 and therefore it is believed that TGM2 is necessary for CLIC3 to drive tumour cell invasiveness (49).

Our data was critical in confirming that CLIC3 can act as an oxidoreductase and thus it also seems CLIC3 and TGM2 interact in a way that depends on the redox environment. Together with our collaborators we have shown that CLIC3 achieves its pro-invasive functions by acting as a redox enzyme to activate TGM2 (49). The group at Glasgow went on to demonstrate that CLIC3 may also influence TGM2 capacity to bind to integrins, which may contribute to the α5β1-dependence of CLIC3 driven invasiveness of tumour cells (49).

Therefore, our data indicates that CLIC3 interacts in a way that would be dependent upon the redox environment, the 1mM of reduced glutathione or GSH in the HEDS assay solution keeps the environment reduced to influence the consumption of the NADPH in the HEDS assay. Also, our CLICs proteins are aliquoted and stored in column sizing buffer (100mM KCl, 1mM NaN3, and 20mM HEPES pH 7.5; containing 0.5mM TCEP) to keep the proteins in a reduced monomeric form. Future studies could explore the influence of pH and redox that mimic different intra-and extracellular locations of each protein. Preliminary studies in our lab indicate that various CLIC proteins have optimal activity based on pH and temperature (54)(*Ms Saba Moghaddasi, MSc Thesis, UTS 2018*).

The protein CLIC3 was first identified in a yeast two hybrid screen, where it was found to bind to ERK7 (67). ERK7 is part of the mitogen-activated protein kinase family of signal transducers. CLIC3 interacted with the COOH-domain of ERK7, which was used as a bait. Qian and colleagues (67) showed via Northern blot analysis that CLIC3 is expressed in the heart, the lung and the placenta. Finally, they showed the association of

ERK7 with CLIC3 via co-immunoprecipitation and found that the protein, like CLIC1, is mainly localised to the nucleus, but it was also detected in the cell cytoplasm (67).

In 2010 the crystal structure of CLIC3 was resolved at 2 Å resolution by Littler and colleagues (Figure 2.21) (68). The crystal structure of CLIC3 revealed that it can be present in the oxidised and reduced state. CLIC3 was identified as being monomeric in solution. In addition, it possesses a GST like form, with a more open and polar active site compared to the other CLIC member proteins. This suggests the possibility that it forms distinct interactions with other proteins during specific cellular processes. The foot loop region of CLIC3 was shown to be more flexible compared to the other CLICs, which taken together might suggest that CLIC3 is functionally divergent (68).

As previously mentioned, that GST-fold in the CLICs consists of two domains: The C-terminal all helical domain and an N-terminal thioredoxin-like domain. The N-terminal thioredoxin domain contains the active site, which in CLIC3 is comprised of two active cysteines. CLIC3 is therefore able to form an internal disulphide bond within it thioredoxin-like CXXC motif (Figure 2.3) (68). This cysteine motif is homologous to that of the GST-fold and likely serves to fine-tune the reactivity and enzymatic action towards the substrate. Moreover, the soluble GST-like state of the CLICs has been well characterised, but the structural transition into a membrane spanning ion channel remains less well characterised. In CLIC3 a disulfide bond may form between the CXXC motif Cysteine 22 and Cysteine 25 (Figure 2.22). With the formation of a disulfide bond it could reduce other proteins which contain a disulphide bond, thus fulfilling its role as an oxidoreductase enzyme (49).

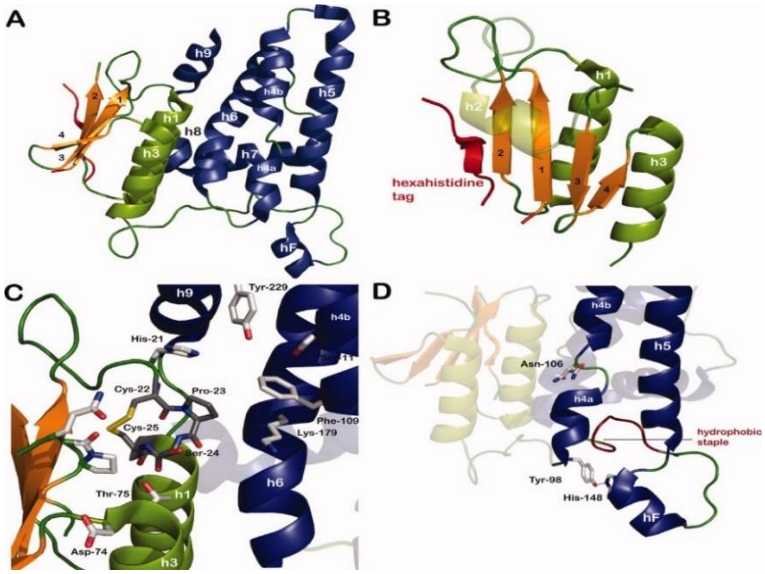

Figure 2. 15 Cartoon representation of CLIC3: A - loop regions are coloured dark green, β-strands orange, and α-helices light green within the N-terminal domain or blue within the C-terminal domain. *All secondary structural elements are labelled. B: A similar representation of the N-terminal thioredoxin domain alone (residues 5–88), red highlights the hexahistidine tag attached to the β-sheet. Also shown is a transparent depiction of helix 2 within the position in which it is observed in CLIC3. This entire segment is disordered in this structure. C: The residues within the CLIC3 active site whose function is currently unclear, the region displayed is analogous to glutathione binding and substrate binding regions of the GSTs. D: The ordered CLIC3 foot loop and the split helix 4 (68).*

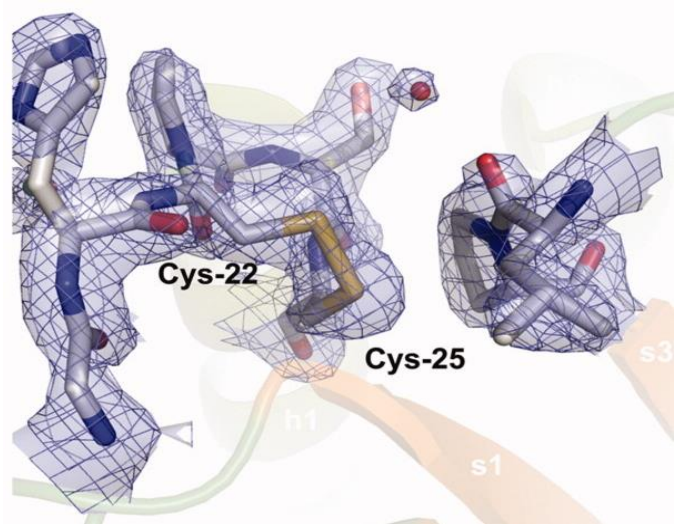

Figure 2. 16 Conserved cysteines in CLIC3 crystallised in the reduced state. Electron cloud of the potential active site of CLIC3 protein. *A potential disulfide bond can form between the two cysteines. Adapted from Littler and colleagues (68).*

Certain extracellular enzymes possess cysteine residues which control their activity, and reduction of oxidized cysteines in extracellular proteins is known to be performed by thioredoxins which use NADH as a source of reducing equivalents. Like thioredoxins, glutathione transferases catalyse reduction of target cysteines using glutathione as a source of reducing equivalents (69). The glutaredoxin-like activity requires an active site cysteine. In the G-site motif of the glutaredoxin system, the first cysteine residue is reported to attack sulfur atoms in disulfide bridges - as occurs within glutathione mixed disulfide bonds - and therefore promotes thiol transfer (56). We could also expect that members of the CLIC family are capable of carrying out targeted protein de/glutathionylation activity. This is supported by the X-ray crystallographic studies that reveal an open slot adjacent to the GSH binding site in CLIC1 that is large enough to accommodate a protein substrate (37). Therefore, de/glutathionylation may well be a

mechanism by which CLIC proteins control ion channel activity and other cellular processes (47, 70).

Our mutagenesis results showed that Cys24 in CLIC1 is the essential catalytic cysteine, as expected from the CLIC1 structure (37). This confirms that the Cys24 in CLIC1 is essential for the enzymatic function of CLIC1 (Figure 2.16), while both Cys 22 and 25 are needed for optimal CLIC3 oxidoreductase activity. The findings in this project supported that both Cys 22, 25 are the key active cysteine residues involved in CLIC3 oxidoreductase activity and are necessary for its optimal enzymatic functions (Figure 2.17), given that the single Cys22 mutant, did maintain some activity however it was greatly diminished compared to wild type CLIC3.

Plant dehydroascorbate reductases (DHAR) adopt a three-dimensional structure similar to the soluble form of CLIC1 (71, 72). It has been noted that the protein PtoDHAR2 from *Populus tomentose* plant, demonstrates oxidoreductase activity and that mutation of its Cys20 residue which is located in the predicted GSH binding site led to abolishment of its reductase activity (71). These predictions are closely related with our results for the CLIC1 mutant (C24A) (Figure 2.16) and the CLIC3 mutant (C22,25A) (Figure 2.17), where they were inactive in the HEDS enzyme assay.

Interestingly, it was postulated that the only two positively charged residues: Arginine 29 (Arg29) and lysine 37 (Lys37) located in the putative transmembrane region of CLIC1 protein (60), and which are also arranged in close vicinity to the enzymatic active site (see figure 2.13), are likely to have a key role in the enzymatic functional activity of CLIC1. These same two residues have previously been shown to regulate the ion channel activity of CLIC1 (60). Our group has also shown that substituting the glycine residues at positions 18 or 22 to alanine in CLIC1 significantly reduces the oxidoreductase activity of CLIC1 proteins . The functional activity of G18A and G22A CLIC1 mutants assessed

using the HEDS assay, showed that the both G18A and G22A CLIC1 mutants were capable of reducing the HEDS substrate in the presence of glutathione reductase (GR). However, both G18A and G22A CLIC1 mutants showed reduced enzymatic activity in comparison to that of CLIC1-wt (59)(*Ms, Khondker Rufaka Hossain PhD Thesis, UTS 2016)*. Therefore, it is clear that specific amino acids within and adjacent to the active site, are critical for optimal and specific enzymatic activity. Similarly, we have noted that the addition of positively charged Histidine residues (His-tag) at the amino terminus of the protein also impact upon the activity of the CLIC proteins (work done by *Ms Saba Moghaddasi, MSc Thesis 2018*). Crystal structures by the Curmi group at UNSW have indicated that the His-tag would fold and come into close proximity to the CLIC enzyme G-site, thus there would be a substantial charge effect via the six Histidine residues upon the redox sensitive Cysteine (unpublished data).

Therefore, in this study in order to determine the functional effects of mutating the only two positively charged residues in the vicinity of the G-site in CLIC1: Arginine 29 (Arg 29) and Lysine 37 (Lys 37) were mutated to neutral Alanine (Ala). The enzymatic function of these mutants was determined in the HEDS enzyme assay (Figure 2.14). Both R29A and K37A CLIC1 mutants were still capable of reducing the HEDS substrate, however their activity was reduced when compared to that of CLIC1-wt. The two mutant versions CLIC1-K37A, and CLIC1-R29A were found to have slower kinetic profiles, thus slower relative activity compared to wild type CLIC1 in particular at the latter stages of the assay, over a 40-minute period (Figure 2.15). Hence, not surprisingly, this indicates that the environment immediately surrounding the active cysteine contributes to the recognition and binding of CLIC substrates and/ or binding partners. Although the CLIC family members have highly conserved active sites, with all 6 members conserving both

K37 and R29 (numbering from CLIC1), this may suggest they would bind similar substrates (68), however there remain several distinct differences in the vicinity of the G-site, which could be critical in defining the substrate specificity of each member.

In addition, mutation of these two charged residues, Arginine 29 and Lysine 37 to the more hydrophobic amino acid Alanine (Ala) would likely create a more hydrophobic cleft near the active cysteine, that could impact on the openness of the active site itself. Due to the proximity and close distance of these two residues to the active site Cysteine 24, we speculate that the substitution of these two residues to neutral and hydrophobic alanine could directly alter the structure or hinder the interaction of substrates such as target proteins, and thus impact on the functional activity of CLIC1. This could further explain the difference in activity seen in our experimental studies. Since the assays were carried out with the purified proteins in solution, additional structural analysis for the CLIC1 proteins by techniques such as X-ray crystallography, NMR and circular dichroism are now required to determine the effect of such single point mutations on the enzyme active site and hence, on the functional activity of CLIC1 and by extension, the other CLIC family members.

Moreover, invertebrates also possess CLIC-like proteins. The nematode *Caenorhabditis elegans* is known to contain two CLIC homologues, EXC-4 and EXL-1 (EXC-4 like protein 1) (73). However, they appear to have a distinct active site to the human CLICs, with the consensus sequence for the invertebrate CLIC-like proteins being: [Cys/Asp] - Leu-Phe-Cys-Gln-Glu. Our results showed that the CLIC like protein Exc-4 showed an apparent lower oxidoreductase activity compared to human CLIC1 in the HEDS enzyme assay (Figure 2.15). This could suggest that the invertebrate CLIC-like proteins have evolved away from the putative enzymatic function of the vertebrate CLICs with their

membrane activity predominating. This suggestion is also supported by the previous work of Berry et al., (2003) who demonstrated that exc-4 localises predominantly to cellular membranes and its role is critical for unicellular tubulogenesis, via regulation of hydrostatic pressure or vesicular dynamics.

Taken together, our data indicates that members of the human CLIC protein family, now including CLIC3, which are well known to function as ion channels when integrated into membranes (74, 75), also demonstrate glutaredoxin-like enzymatic activity when in their soluble form. This supports an additional role for these proteins in cellular processes such as detoxification and oxidoreduction.

2.11. 4 Chloride Ion Channel Blockers Inhibit the Enzymatic Activity of both CLIC1and CLIC3

An intriguing finding of our work was the inhibitory effect of the chloride ion channel blocker, IAA-94 on the enzymatic activity of CLIC1-wt (Figure 2.18) and CLIC3-wt (Figure 2.19). The IAA-94 drug was able to completely block the enzymatic activity of CLIC1-wt and CLIC3-wt in the HEDS assay. Our findings are consistent with the structural and evolutionary relationship between the GST and CLIC families, as IAA-94 is a homologue of the GST enzyme inhibitor ethacrynic acid (76, 77) a point also noted during the determination of the structure of CLIC1 (37). Electrophysiological experiments have previously demonstrated that CLIC1 channel activity can be blocked by IAA-94 and A9C but not by DIDS (61). The growth of CHOK1 cells, in the presence of IAA-94 and A9C, was blocked at G2M phase of the cell cycle (61).

Given that these drugs are membrane permeable and are capable of entering inside the cells, one could speculate that these previously attributed cellular inhibitory effects, such as arrest of the cell cycle progression are not exclusively due to blocking of the ion channel activity but could equally also be due to inhibition of CLIC protein enzymatic activity.

This concurrence of enzymatic inhibitory profile and channel function blockage has profound consequences. The structural transition of CLIC1 from its soluble form to its integral membrane form likely results in a complete disruption of its thioredoxin-like N-terminal domain (37, 51, 75, 78, 79). Thus, if IAA-94 binds to the soluble form of CLIC1 in the cleft between the N-domain and the C-domain, as has been reported for the GST proteins (80, 81) then it is unlikely to bind to this same site on the integral membrane form, a this binding site would no longer exist. It has in fact been suggested that the inhibitor IAA-94 binds instead to a new, distinct site on the integral membrane form of CLIC1 (81). Another explanation offered, is that the inhibition of the channel is mediated by inhibition of the enzymatic activity of the soluble form of CLIC1. Binding of the inhibitors to a new, distinct site on the CLIC proteins seems unlikely, albeit possible. A more likely explanation for their ability to inhibit both functions is that the inhibitors, such as IAA-94 and A9C, act by binding, presumably near the active site of the soluble form of CLIC1, thus inhibiting its enzymatic activity and as a consequence, its channel activity. This suggestion however remains speculative, requiring further investigation in order to determine the precise mechanism of action.

2. 12 Concluding Remarks

Members of the CLIC protein family, which are known to function as ion channels when integrated into membranes, also demonstrate glutaredoxin-like enzymatic activity when in their soluble form (as described in this, Chapter 2). This supports an additional role for these proteins in such cellular processes, as detoxification and oxidoreduction. Also, the fact that the same CLIC protein channel blockers inhibit CLIC1 and CLIC3 enzymatic function, suggests that the enzymatic properties of CLIC1 and CLIC3 may also control the function of the channel form of these proteins.

The remainder of this thesis aims to elucidate further the role of the CLIC proteins within cells, focusing on their oxidoreductase enzymatic activity, that is likely serving a protective role against oxidative stress (Chapter 3). Chapter 4 aims covers work investigating the role of the CLIC proteins in glutathionylation and/or deglutathionylation reactions, with the longer vision being identification of specific protein targets.

Overall, the results obtained from the experiments presented in this Chapter 2, demonstrate that the Chloride Intracellular Ion Channel Protein 3, also functions as an Oxidoreductase Enzyme like the other CLIC members (namely CLIC1, 2 and 4).

Based upon these findings, our collaborators have demonstrated an extracellular target of CLIC3 enzymatic activity is the protein Transglutaminase 2 (TG2) Which is physically associates with CLIC3 and that TGM2 is necessary for CLIC3 to drive tumour cell invasiveness (49).

Pursuit of other functions of the soluble form of the CLICs, in particular protective roles in cells against oxidative damage or as participants in the cellular glutathionylation cycle is the focus of the subsequent chapters of this thesis.

2. 13 References

1. **Hsu PP, Sabatini DM.** 2008. Cancer cell metabolism: Warburg and beyond. Cell **134:**703-707.
2. **Bergareche AM, Ruiz-Mirazo K.** 1999. Metabolism and the problem of its universalization. BioSystems **49:**45-61.
3. **Vander Heiden MG, Cantley LC, Thompson CB.** 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. science **324:**1029-1033.
4. **Cooke MS, Evans MD, Dizdaroglu M, Lunec J.** 2003. Oxidative DNA damage: mechanisms, mutation, and disease. The FASEB Journal **17:**1195-1214.
5. **Matés JM, Segura JA, Alonso FJ, Márquez J.** 2010. Roles of dioxins and heavy metals in cancer and neurological diseases using ROS-mediated mechanisms. Free Radical Biology and Medicine **49:**1328-1341.
6. **Melis JP, van Steeg H, Luijten M.** 2013. Oxidative DNA damage and nucleotide excision repair. Antioxidants & redox signaling **18:**2409-2419.
7. **Storr SJ, Woolston CM, Zhang Y, Martin SG.** 2013. Redox environment, free radical, and oxidative DNA damage. Antioxidants & redox signaling **18:**2399-2408.

8. **Covarrubias L, Hernández-García D, Schnabel D, Salas-Vidal E, Castro-Obregón S.** 2008. Function of reactive oxygen species during animal development: passive or active? Developmental biology **320:**1-11.

9. **Suttorp N, Toepfer W, Roka L.** 1986. Antioxidant defense mechanisms of endothelial cells: glutathione redox cycle versus catalase. American Journal of Physiology-Cell Physiology **251:**C671-C680.

10. **Qanungo S, Starke DW, Pai HV, Mieyal JJ, Nieminen A-L.** 2007. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFκB. Journal of Biological Chemistry **282:**18427-18436.

11. **Wang J, Boja ES, Tan W, Tekle E, Fales HM, English S, Mieyal JJ, Chock PB.** 2001. Reversible glutathionylation regulates actin polymerization in A431 cells. Journal of Biological Chemistry **276:**47763-47766.

12. **Owen JB, Butterfield DA.** 2010. Measurement of oxidized/reduced glutathione ratio, p 269-277, Protein Misfolding and Cellular Stress in Disease and Aging. Springer.

13. **Miwa S, Beckman KB, Muller F.** 2008. Oxidative stress in aging: from model systems to human diseases. Springer Science & Business Media.

14. **Holmgren A.** 2000. Antioxidant function of thioredoxin and glutaredoxin systems. Antioxidants & redox signaling **2:**811-820.

15. **Trotter EW, Grant CM.** 2003. Non-reciprocal regulation of the redox state of the glutathione–glutaredoxin and thioredoxin systems. EMBO reports **4:**184-188.

16. **Wood ZA, Schröder E, Harris JR, Poole LB.** 2003. Structure, mechanism and regulation of peroxiredoxins. Trends in biochemical sciences **28:**32-40.

17. **Katti SK, LeMaster DM, Eklund H.** 1990. Crystal structure of thioredoxin from Escherichia coli at 1.68 Å resolution. Journal of molecular biology **212:**167-184.

18. **Arnér ES, Holmgren A.** 2000. Physiological functions of thioredoxin and thioredoxin reductase. European Journal of Biochemistry **267:**6102-6109.

19. **Akterin S, Cowburn RF, Miranda-Vizuete A, Jiménez A, Bogdanovic N, Winblad B, Cedazo-Minguez A.** 2006. Involvement of glutaredoxin-1 and thioredoxin-1 in β-amyloid toxicity and Alzheimer's disease. Cell death and differentiation **13:**1454.

20. **Holmgren A, Lu J.** 2010. Thioredoxin and thioredoxin reductase: current research with special reference to human disease. Biochemical and biophysical research communications **396:**120-124.

21. **Du Y, Zhang H, Zhang X, Lu J, Holmgren A.** 2013. Thioredoxin 1 is inactivated due to oxidation induced by peroxiredoxin under oxidative stress and reactivated by glutaredoxin system. Journal of Biological Chemistry**:**jbc. M113. 495150.

22. **Watson WH, Pohl J, Montfort WR, Stuchlik O, Reed MS, Powis G, Jones DP.** 2003. Redox potential of human thioredoxin 1 and identification of a second dithiol/disulfide motif. Journal of Biological Chemistry **278:**33408-33415.

23. **Chrestensen CA, Eckman CB, Starke DW, Mieyal JJ.** 1995. Cloning, expression and characterization of human thioltransferase (glutaredoxin) in E. coli. FEBS letters **374:**25-28.

24. **Gallogly MM, Starke DW, Leonberg AK, Ospina SME, Mieyal JJ.** 2008. Kinetic and mechanistic characterization and versatile catalytic properties of mammalian glutaredoxin 2: implications for intracellular roles. Biochemistry **47:**11144-11157.

25. **Holmgren A.** 1979. Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli. Journal of Biological Chemistry **254:**3664-3671.

26. **Holmgren A, Aslund F.** 1995. [29] Glutaredoxin, p 283-292, Methods in enzymology, vol 252. Elsevier.

27. **Herrero E, De La Torre-Ruiz M.** 2007. Monothiol glutaredoxins: a common domain for multiple functions. Cellular and molecular life sciences **64:**1518-1530.

28. **Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, McConnell J.** 2010. Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. PloS one **5:**e10074.

29. **Bushweller JH, Aaslund F, Wuethrich K, Holmgren A.** 1992. Structural and functional characterization of the mutant Escherichia coli glutaredoxin (C14. fwdarw. S) and its mixed disulfide with glutathione. Biochemistry **31:**9288-9293.

30. **Sagemark J, Elgán TH, Bürglin TR, Johansson C, Holmgren A, Berndt KD.** 2007. Redox properties and evolution of human glutaredoxins. Proteins: Structure, Function, and Bioinformatics **68:**879-892.

31. **Yang Y, Wells WW.** 1991. Identification and characterization of the functional amino acids at the active center of pig liver thioltransferase by site-directed mutagenesis. Journal of Biological Chemistry **266:**12759-12765.

32. **Mashamaite LN, Rohwer JM, Pillay CS.** 2015. The glutaredoxin mono-and di-thiol mechanisms for deglutathionylation are functionally equivalent: implications for redox systems biology. Bioscience reports **35:**e00173.

33. **Lillig CH, Prior A, Schwenn JD, Åslund F, Ritz D, Vlamis-Gardikas A, Holmgren A.** 1999. New thioredoxins and glutaredoxins as electron donors of 3′-

phosphoadenylylsulfate reductase. Journal of Biological Chemistry **274:**7695-7698.

34. **Toledano MB, Kumar C, Le Moan N, Spector D, Tacnet F.** 2007. The system biology of thiol redox system in Escherichia coli and yeast: differential functions in oxidative stress, iron metabolism and DNA synthesis. FEBS letters **581:**3598-3607.

35. **Hayes JD, Flanagan JU, Jowsey IR.** 2005. Glutathione transferases. Annu Rev Pharmacol Toxicol **45:**51-88.

36. **Wilce MCaMWP.** 1994. Structure and function of glutathione S-transferases. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology **1205:**1-18.

37. **Harrop SJ, DeMaere MZ, Fairlie WD, Reztsova T, Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Jankova L, Warton K.** 2001. Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-Å resolution. Journal of Biological Chemistry **276:**44993-45000.

38. **Kanaoka Y, Ago H, Inagaki E, Nanayama T, Miyano M, Kikuno R, Fujii Y, Eguchi N, Toh H, Urade Y.** 1997. Cloning and crystal structure of hematopoietic prostaglandin D synthase. Cell **90:**1085-1095.

39. **Collet J-F, Messens J.** 2010. Structure, function, and mechanism of thioredoxin proteins. Antioxidants & redox signaling **13:**1205-1216.

40. **Garcerá A, Barreto L, Piedrafita L, Tamarit J, Herrero E.** 2006. Saccharomyces cerevisiae cells have three Omega class glutathione S-transferases acting as 1-Cys thiol transferases. Biochemical Journal **398:**187-196.

41. **Gravina SA, Mieyal JJ.** 1993. Thioltransferase is a specific glutathionyl mixed-disulfide oxidoreductase. Biochemistry **32:**3368-3376.

42. **He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, Da Costa LT, Morin PJ, Vogelstein B, Kinzler KW.** 1998. Identification of c-MYC as a target of the APC pathway. Science **281:**1509-1512.

43. **RAGHAVACHARI N, QIAO F, LOU MF.** 1999. Does glutathione-S-transferase dethiolate lens protein-thiol mixed disulfides?—A comparative study with thioltransferase. Experimental eye research **68:**715-724.

44. **Mieyal JJ, Gallogly, M. M., Qanungo, S., Sabens, E. A., & Shelton, M. D. .** 2008. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. . Antioxidants and Redox Signaling, **10** 1941-1988.

45. **Dulhunty A, Gage P, Curtis S, Chelvanayagam G, Board P.** 2001. The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. Journal of Biological Chemistry **276:**3319-3323.

46. **Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV.** 2000. Identification, characterization, and crystal structure of the Omega class glutathione transferases. Journal of Biological Chemistry **275:**24798-24806.

47. **Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, Valenzuela SM, Mazzanti M, Brown LJ, Breit SN.** 2010. The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? FEBS letters **584:**2093-2101.

48. **Al Khamici H, Brown LJ, Hossain KR, Hudson AL, Sinclair-Burton AA, Ng JPM, Daniel EL, Hare JE, Cornell BA, Curmi PM.** 2015. Members of the Chloride Intracellular Ion Channel Protein Family Demonstrate Glutaredoxin-Like Enzymatic Activity. PloS one **10:**e115699.

49. **Hernandez-Fernaud JR, Ruengeler E, Casazza A, Neilson LJ, Pulleine E, Santi A, Ismail S, Lilla S, Dhayade S, MacPherson IR.** 2017. Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity. Nature communications **8:**14206.

50. **Froger A, Hall JE.** 2007. Transformation of plasmid DNA into E. coli using the heat shock method. Journal of visualized experiments: JoVE.

51. **Littler DR, Harrop SJ, Fairlie WD, Brown LJ, Pankhurst GJ, Pankhurst S, DeMaere MZ, Campbell TJ, Bauskin AR, Tonini R.** 2004. The intracellular chloride ion channel protein CLIC1 undergoes a redox-controlled structural transition. Journal of Biological Chemistry **279:**9298-9305.

52. **Block H, Maertens B, Spriestersbach A, Brinker N, Kubicek J, Fabis R, Labahn J, Schäfer F.** 2009. Immobilized-metal affinity chromatography (IMAC): a review, p 439-473, Methods in enzymology, vol 463. Elsevier.

53. **Sotirchos IM, Hudson AL, Ellis J, Davey MW.** 2009. A unique thioredoxin of the parasitic nematode Haemonchus contortus with glutaredoxin activity. Free Radical Biology and Medicine **46:**579-585.

54. **Moghaddasi S.** 2018. Biochemical characterisation of the enzymatic activity of chloride intracellular ion channel proteins.

55. **Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry **72:**248-254.

56. **Holmgren A.** 1995. Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. Structure **3:**239-243.

57. **Sotirchos IM HA, Ellis J, Davey MW.** 2009. . A unique thioredoxin of the parasitic nematode Haemonchus contortus with glutaredoxin activity. . Free Radical Biology and Medicine **46:**:579-585.

58. **Valenzuela SM, Martin DK, Por SB, Robbins JM, Warton K, Bootcov MR, Schofield PR, Campbell TJ, Breit SN.** 1997. Molecular cloning and expression of a chloride ion channel of cell nuclei. Journal of Biological Chemistry **272:**12575-12582.

59. **Hossain KR.** 2016. A study of the spontaneous membrane insertion of chloride intracellular ion channel protein CLIC1 into model lipid membranes.

60. **Averaimo S, Abeti R, Savalli N, Brown LJ, Curmi PM, Breit SN, Mazzanti M.** 2013. Point mutations in the transmembrane region of the CLIC1 ion channel selectively modify its biophysical properties. PloS one **8:**e74523.

61. **Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Warton K, Musgrove EA, Campbell TJ, Breit SN.** 2000. The nuclear chloride ion channel NCC27 is involved in regulation of the cell cycle. The Journal of physiology **529:**541-552.

62. **Björnstedt M, Kumar S, Holmgren A.** 1994. Selenite and selenodiglutathione: reactions with thioredoxin systems. Methods in enzymology **252:**209-219.

63. **Tulk BM, Kapadia S, Edwards JC.** 2002. CLIC1 inserts from the aqueous phase into phospholipid membranes, where it functions as an anion channel. American Journal of Physiology-Cell Physiology **282:**C1103-C1112.

64. **Mesecke N, Spang A, Deponte M, Herrmann JM.** 2008. A novel group of glutaredoxins in the cis-Golgi critical for oxidative stress resistance. Molecular biology of the cell **19:**2673-2680.

65. **Liao B-C, Hsieh C-W, Lin Y-C, Wung B-S.** 2010. The glutaredoxin/glutathione system modulates NF-κB activity by glutathionylation of p65 in cinnamaldehyde-treated endothelial cells. Toxicological sciences **116:**151-163.

66. **Jin X, Stamnaes J, Klöck C, DiRaimondo TR, Sollid LM, Khosla C.** 2011. Activation of extracellular transglutaminase 2 by thioredoxin. Journal of Biological Chemistry**:**jbc. M111. 287490.

67. **Qian Z, Okuhara D, Abe MK, Rosner MR.** 1999. Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. Journal of Biological Chemistry **274:**1621-1627.

68. **Littler D, Brown L, Breit S, Perrakis A, Curmi P.** 2010. Structure of human CLIC3 at 2 Å resolution. Proteins: Structure, Function, and Bioinformatics **78:**1594-1600.

69. **Wang L, He S, Tu Y, Ji P, Zong J, Zhang J, Feng F, Zhao J, Zhang Y, Gao G.** 2012. Elevated expression of chloride intracellular channel 1 is correlated with poor prognosis in human gliomas. J Exp Clin Cancer Res **31:**44.

70. **Jiang L, Phang JM, Yu J, Harrop SJ, Sokolova AV, Duff AP, Wilk KE, Alkhamici H, Breit SN, Valenzuela SM.** 2014. CLIC proteins, ezrin, radixin, moesin and the coupling of membranes to the actin cytoskeleton: a smoking gun? Biochimica et Biophysica Acta (BBA)-Biomembranes **1838:**643-657.

71. **Tang Z-X, Yang H-L.** 2013. Functional divergence and catalytic properties of dehydroascorbate reductase family proteins from Populus tomentosa. Molecular biology reports **40:**5105-5114.

72. **Elter A, Hartel A, Sieben C, Hertel B, Fischer-Schliebs E, Lüttge U, Moroni A, Thiel G.** 2007. A plant homolog of animal chloride intracellular channels

(CLICs) generates an ion conductance in heterologous systems. Journal of Biological Chemistry **282:**8786-8792.

73. **Littler DR, Harrop SJ, Brown LJ, Pankhurst GJ, Mynott AV, Luciani P, Mandyam RA, Mazzanti M, Tanda S, Berryman MA.** 2008. Comparison of vertebrate and invertebrate CLIC proteins: The crystal structures of Caenorhabditis elegans EXC-4 and Drosophila melanogaster DmCLIC. Proteins: Structure, Function, and Bioinformatics **71:**364-378.

74. **Edwards JC, Kahl CR.** 2010. Chloride channels of intracellular membranes. FEBS letters **584:**2102-2111.

75. **Singh H.** 2010. Two decades with dimorphic chloride intracellular channels (CLICs). FEBS letters **584:**2112-2121.

76. **Landry DW, Akabas MH, Redhead C, Edelman A, Cragoe EJ, Al-Awqati Q.** 1989. Purification and reconstitution of chloride channels from kidney and trachea. Science **244:**1469-1472.

77. **Ploemen J, Ommen BV, Bogaards J, Van Bladeren P.** 1993. Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione S-transferases. Xenobiotica **23:**913-923.

78. **Goodchild SC, Howell MW, Cordina NM, Littler DR, Breit SN, Curmi PM, Brown LJ.** 2009. Oxidation promotes insertion of the CLIC1 chloride intracellular channel into the membrane. European Biophysics Journal **39:**129-138.

79. **Goodchild SC, Howell MW, Littler DR, Mandyam RA, Sale KL, Mazzanti M, Breit SN, Curmi PM, Brown LJ.** 2010. Metamorphic response of the CLIC1 chloride intracellular ion channel protein upon membrane interaction. Biochemistry **49:**5278-5289.

80. **Oakley AJ, Rossjohn J, Lo Bello M, Caccuri AM, Federici G, Parker MW.** 1997. The three-dimensional structure of the human Pi class glutathione transferase P1-1 in complex with the inhibitor ethacrynic acid and its glutathione conjugate. Biochemistry **36:**576-585.

81. **Cameron AD, Sinning I, L'Hermite G, Olin B, Board PG, Mannervik B, Jones TA.** 1995. Structural analysis of human alpha-class glutathione transferase A1-1 in the apo-form and in complexes with ethacrynic acid and its glutathione conjugate. Structure **3:**717-727.

Chapter 3

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3. 1 Introduction

3.1. 2 Physiological Role of Reactive Oxygen Species (ROS)

Cellular oxidative stress arises when there is excessive production of Reactive Oxygen Species (ROS) that overwhelm the cell's anti-oxidant systems (1). High levels of ROS are very toxic to organisms as they need to pair with other molecules to gain stability, consequently, attacking many biomolecules such as lipids, nucleic acids, proteins and carbohydrates resulting in various levels of biological damage (2). As such, throughout evolution, as cells were exposed to an increasingly oxidizing environment, they found it necessary to adapt to these conditions in order to maintain a reduced intracellular state and develop means of defending against ROS.

ROS however are also important molecules in a number of physiological processes. Their reactivity is the foundation for their functions, in that it renders the possibility for them to interact with other molecules. The physiological role of ROS can be divided into two effects: firstly, a killing effect and secondly, a signalling effect.

3.1.1. 4 The Signalling Effect of ROS

For many years, ROS were considered simply as undesired toxic metabolites, causing cell damage. Surprisingly, during the last few decades, they have been increasingly recognized as messenger molecules mediating redox signalling. A large part of ROS-mediated signal transduction relies on the post-translational modification of cysteine thiols (-SH) within proteins (3). Several properties qualify thiols as signal switches. First of all, thiols are susceptible to ROS challenge that lays a foundation for its sensitivity. Secondly, due to the different local environment, thiols exhibit different reactivity which renders them a level of selectivity (4). Lastly, most of the thiol

modifications are reversible which provides flexibility for sophisticated signalling regulation.

The major redox signalling molecules in cells are H_2O_2 and NO. H_2O_2 can lead to reversible thiol modifications such as S-sulfenylation (sulfenic acid, -SOH), sulfinic acid ($-SO_2H$), disulfide bond (-S-S-), and S-glutathionylation (-SSG) when glutathione (GSH) is present. If sulfinic acid gets further oxidized, sulfonic acid ($-SO_3H$), an irreversible thiol modification, will be formed and it has been considered as a hallmark of diseases and usually leads to permanent protein functional inactivation and protein degradation (5).

3.1.1. 5 Cellular Damage by ROS

Excessive ROS reacts directly with biological molecules such as lipids, DNA, and proteins and cause damages to them. For example, biological membranes contain polyunsaturated fatty acid to maintain their functions and fluidity (6). However, unsaturated lipid is sensitive to ROS which produces lipid peroxides, disrupts normal membrane structure of cells and organelles, such as mitochondria and Endoplasmic Reticulum, and results in loss of function and changes to enveloped contents, finally resulting in necrotic cell death (7). Both nuclear and mitochondrial DNA can be attacked by ROS and several types of DNA lesions have been observed including oxidation of sugar and base moieties, DNA single strand breaks (8) and double strand breaks (9) which then trigger apoptosis – programmed cell death (10).

3.1.1. 6 Signalling Dysfunction of ROS

Redox signalling is also affected by oxidative stress and the dysfunction is reflected in aberrant protein thiol modifications. Usually, when DNA damage happens, the genome guardian, cellular tumour antigen p53 gets activated as a transcription factor and

triggers multiple downstream effects, such as DNA repair, cell cycle arrest, and apoptosis, so cells with damaged DNA will not transform into cancer cells (11). When there are high level of ROS, such as occurs in times of inflammation, cysteine residues in the DNA binding site of p53 can be abnormally S-glutathionylated (12). This redox modification of p53 leads to its loss of surveillance function. In cancer this means aberrant cells are no longer triggered to become apoptotic, while in neurodegenerative diseases, increased neuronal cell death is caused by the elevated oxidative stress.

3. 2 Antioxidant Systems in Cells

In order to maintain redox balance and protect the cells from the harmful effect of ROS, aerobic organisms have developed antioxidant defences which either eliminate these species or prevent their formation. The amount of reactive species inside the cells is maintained through redox homeostasis, avoiding oxidative damage. A decrease in antioxidant defence or an excess production of reactive oxygen species will lead to an imbalance and may potentially lead to oxidative damage. The antioxidant is "any substance which significantly delays, prevents oxidation or removes oxidative damage to a target molecule" (13). There are a wide range of antioxidants, each with different targets, mechanisms and efficiencies, some of which cooperate together (14).

The thioredoxin system, which is comprised of thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, is a major cellular disulfide reduction system targeting a broad range of substrates. After reducing its targets, a disulfide is formed in Trx that is subsequently reduced by TrxR using NADPH as the electron donor. Thioredoxin is a ubiquitous small enzyme around 12-kDa with a (Cysteine32 -Glycine-Proline-Cysteine35) motif at its active site, which was first discovered in *Escherichia coli* to provide electrons for ribonucleotide reductase (RNR), an essential enzyme in the

process of DNA synthesis, responsible for catalysing the formation of deoxyribonucleotides from ribonucleotides (15, 16). The 3D structure of bacterial Trx was the first described by Holmgren in 1975 (17).

Recently, many of the Trxs group superfamily have been structurally resolved (18). In mammalian cells, there are three isoforms of Trxs, Trx1 in the cytosol, Trx2 in mitochondria (19), and a testis specific Trx (20). It has been shown that the Trxs utilize the two cysteines at their active site to perform substrate reduction. The N-terminal cysteine at the active site first attacks the disulfide in substrate proteins and forms an intermedia disulfide between Trx and substrate, then the C-terminal cysteine at the active site takes over and leaves reduced protein substrate and an active-site disulfide in Trx which can be subsequently reduced by TrxR (21).

In addition to the two active site cysteines (Cys32 and Cys35 in *Escherichia coli*), the human Trx1 (hTrx1) has three structural cysteines, Cys62, Cys69 and Cys73, indicating that the human Trx1 has unique biological property. Several studies have shown that the structural cysteines of human Trx1 can be redox-modified and regulated in different redox contexts (16, 22). The Thioredoxin system has been shown to interact with a broad range of proteins not only to maintain the reducing cellular environment by transferring electrons to them, but also to mediate different cellular signalling pathways by regulating cysteine post-translational modifications. Furthermore, proteomic techniques help to identify more about potential targets for the Trx system (16, 22).

Consequently, in addition to the Thioredoxin system, the Glutaredoxin system is another cellular disulfide reductase system existing in most living organisms, which is composed of glutaredoxin (Grx), glutathione reductase (GR) and glutathione (GSH) (16, 23). Grxs are low-molecular-weight proteins, that perform important roles in redox regulation, post-translational modification of target protein substrates and are involved

in iron metabolism. This system was first discovered in *Escherichia coli* bacteria by Holmgren in 1976 as a reductase system for RNR in a mutant *Escherichia coli* that is unable to replicate phage T7 and were shown to lack Trx (24). Purification of *E.coli* Grx to homogeneity demonstrated that it contained a single disulfide in its oxidized form with the unique sequence Cys-Pro-Tyr-Cys (CPYC) (25, 26). Additionally, Grx showed a strong GSH-disulfide transhydrogenase activity apart from its role as a GSH-dependent hydrogen donor for RNR. Consequently, Grx was isolated from calf thymus and purified to homogeneity (27, 28).

The human Glutaredoxins (Grx) have been found in four isoforms and are divided into two groups depending upon the number of cysteine residues at their active site: the dithiol Grxs including Grx1 and Grx2 with a CXXC active site motif and the monothiol Grxs including Grx3 and Grx5 with a CXXS active site motif (16). Human Grx1 with a Cys-Pro-Tyr-Cys (CPYC) active site is mainly present in the cytosol and functions as an antioxidant enzyme. Although the gene of Grx1 does not contain subcellular localization sequence, it is also distributed in the nucleus (29, 30), mitochondrial intermembrane space (31), and secreted into plasma (32). Furthermore, the glutaredoxin proteins showed a protective effect against inflammation (33), cardiovascular hypertrophy (34) and airway hyper responsiveness (35).

In the Grx2 the active site is Cys-Ser-Tyr-Cys (CSYC). The subtle substitution of a proline (as found in Grx1) to serine, distinguishes Grx2's ability to receive electrons from mitochondrial TrxR (36). Grx2 participates in antioxidant processes and is considered a redox sensor (37). Knock-out of Grx2 in mice, leads to early-onset of age-dependent cataract (38), enlarged hearts and high blood pressure (39).

Monothiol Grxs contain a Cys-Gly-Phe-Ser (CGFS) motif at their active site and can be further classified into two groups. Grx3 consists of an N-terminal Trx domain that lacks a redox active motif and two monothiol Grx domains that both harbor the CGFS active site motif and it is also named protein kinase C interacting cousin of thioredoxin (PICOT) (40). Grx3 is located in the cytosol and is important for embryonic development (41). Grx3 is also an iron-sulfur cluster protein with a similar redox-sensor function to Grx2 (42). Additionally, another human monothiol Grx, Grx5 is located in mitochondria and contains a single monothiol Grx domain. Grx5 is evolutionarily conserved in eukaryotes (43). It has been reported that Grx5 is essential for mitochondrial iron-sulfur cluster biogenesis (44). Moreover, patients diagnosed with Grx5 deficiency suffer from anaemia and blood iron overload, which also highlights the importance of Grx5 in iron metabolism (45).

In addition to the crucial role of the glutaredoxins (Grx) as electron donors for RNR and other proteins, the Grx system mediates redox signalling mainly by reversibly catalysing protein S-glutathionylation. Despite the fact that several proteins such as Trxs (16), PDI (46) and sulfiredoxin (47) showed their deglutathionylation activity in different cases, glutaredoxins are considered as the major deglutathionylases due to their high affinity and selectivity for specific glutathionylated proteins (36).

3. 3 Proteins thiols-disulphides metabolism in redox regulation

High levels of ROS, diminished antioxidant defence enzymes and depletion of antioxidants may result in oxidative stress and proteins represent a possible target of oxidative damage. Proteins can undergo different types of oxidation, depending on the amino acid targeted and their oxidation can be reversible or irreversible. Irreversible oxidative modifications of proteins (e.g. carbonylation, nitration, protein cross-linking)

are responsible for protein degradation and aggregation, while reversible oxidative modifications (e.g. S-glutathionylation or nitrosylation) can have a dual role, protecting cysteine from irreversible oxidation and modulating protein functions (redox regulation) (48).

Protein modifications include sulfoxidation, carbonylation and nitration. Sulfoxidation is a reversible oxidation of the amino acid methionine in proteins to methionine sulfoxide which is reduced by the enzyme methionine sulfoxide reductase (49). Carbonylation is an irreversible oxidative modification and different diseases such as Parkinson's disease, Alzheimer's disease, cancer and sepsis are associated with high levels of carbonylated proteins (50).

Cysteine (Cys) is the most chemically reactive natural amino acid in proteins and Cys containing proteins are particularly susceptible to various reversible or irreversible oxidative modifications. As shown in Figure 3.1, a thiol group can undergo different post-translational modifications. Typically, Cys can react with another Cys to form a disulphide bond (-S-S-). A disulphide bond can be formed between two Cys residues within the same protein (intramolecular cross-linking) or between two Cys residues from different proteins (intermolecular cross-linking). Mixed disulphides can also be formed between a protein Cys and the Cys of GSH (a process also termed protein glutathionylation or glutathionylation) or with a Cys (protein cysteinylation). S nitrosylation represents the reaction between a thiol group and RNS to form a nitrosothiol (SNO). Protein thiols can also be oxidized by ROS and RNS to sulfenic acid (Cys-SOH), eventually followed by a second oxidation with formation of sulfinic acid (Cys-SO_2H). Sulfinic acids can be further oxidized to sulfonic acid (Cys-SO_3H).

While most the other forms of oxidation are reversible (that is, the protein's Cys can be reduced back to the free thiol), sulfonylation is an irreversible form of oxidation.

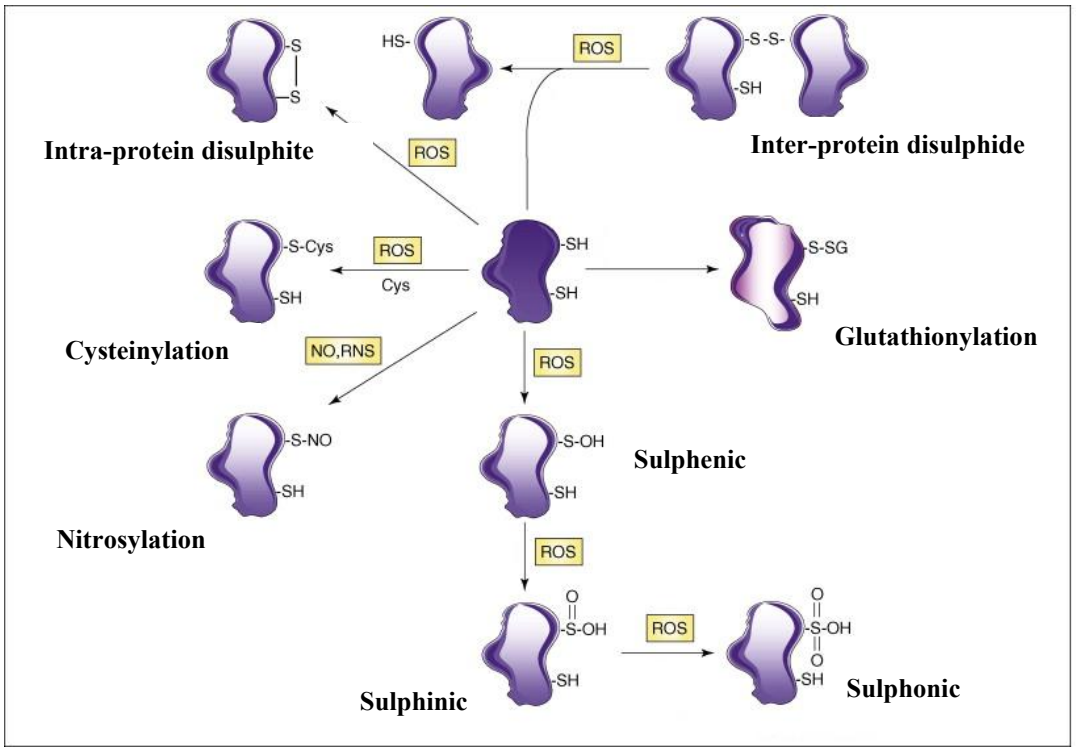

Figure 3. 1 Oxidative modifications of proteins thiol. This was modified from DalleDonne et al. 2009 (51).

In this thesis chapter, we demonstrate for the first time that CLIC proteins possess cellular antioxidant activity. We used H_2O_2, an endogenously existing ROS, to induce oxidative stress in bacterial *E. coli* cells expressing recombinant CLIC proteins in order to investigate if the expression of CLIC proteins by the cells were sufficient to provide increased tolerance to oxidative stress. A mutant form of the various CLIC proteins, lacking the critical cysteine residue in their enzymatic active site were also assessed.

3. 4 Materials and Methods

3.4. 4 Bacterial strains

Transformed bacterial clones overexpressing either CLIC1-wt, CLIC3-wt or CLIC4-wt, along with mutant versions of CLIC1: CLIC1-Cys24S and mutant versions of CLIC3: CLIC1-Cys22,25A were also prepared. Also, corresponding empty vector controls, *E. coli* BL21 (DE3) cells transformed with either pET28a, pGEX-6P-1 and pGEX-2P-T plasmids were used as controls. These were grown and maintained from 50% glycerol stocks, stored at -80°C, as previously described in Section (2.3.1).

3.4. 5 Response of E.coli cells to oxidative stress

To investigate the H_2O_2 tolerance of recombinant CLICs-wt and CLICs mutants in bacterial *E. coli* cells, overnight cultures of *E. coli* BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a, CLIC1-Cys24S in pET28a, plasmids were grown in fresh 2xYT medium containing 30mg/mL Kanamycin antibiotic under continuous shaking condition at 37°C. Also, overnight cultures of *E. coli* BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild-type CLIC3 in pGEX-6P-1, CLIC3-Cys22,25A in pGEX-6P-1, wild-type CLIC4 in pGEX-2P-T, plasmids were grown in fresh 2xYT medium containing 30mg/mL Carbenicillin antibiotic under continuous shaking condition at 37°C. When the A600 nm reached a value of 0.6, the desired concentrations of H_2O_2 (2.5, 5.0 and 10.0 mM) and 1mM IPTG were added simultaneously. The cells were incubated for 3 hours at 20°C with the desired concentrations of H_2O_2 (2.5, 5.0 and 10.0 mM) and 1mM IPTG, then the growth was measured at 600 nm. In addition to measure the effect of these different concentrations of H_2O_2 (2.5, 5.0 and 10.0 mM) on the growth of the bacterial cells at

different time intervals, experiments were run using overnight cultures of *E. coli* BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild-type CLIC3 in pGEX-6P-1, CLIC3-Cys22,25A in pGEX-6P-1, wild-type CLIC4 in pGEX-2P-T, plasmids were grown in fresh 2xYT medium containing 30mg/mL Carbenicillin antibiotic under continuous shaking condition at 37°C. When the A600 nm reached a value of 0.6, the cells were grown and induced with 1mM IPTG at 2 hours at 20°C, then the different concentrations of H_2O_2 (2.5, 5.0 and 10.0 mM) were added. The growth of the bacterial cells was monitored and measured at different time intervals at 20°C over 6 hours of investigation in the absence and presence of 5.0 and 10.0 mM H_2O_2.

3.4. 6 Statistical analysis

Statistical analyses were performed using the software Graph pad Prism 8 excel. Data were analysed by ordinary one-way ANOVA multiple comparisons statistical analysis test. The Data were considered statistically significant when $p<0.05$. Also, we applied the analysis, Area under the curve (AUC) where it means that the Growthcurver computes the area under the logistic curve, which provides a metric (the logistic AUC) that integrates information from the logistic parameters (k, r, and N_0). We implemented this feature by evaluating the definite integral of the fitted logistic equation from time 0 to a user-defined time t. Growthcurver also computes an empirical AUC by summing the areas of the trapezoids made up by connecting consecutive data points of cell counts (or absorbance measurements) from time 0 to time t, similar to what was done by (52).

3. 5 Results

3.5. 2 Antioxidant properties of CLIC Proteins and CLIC Mutant Proteins

In this study, we used *E.coli* BL21 (DE3) bacteria containing the His or GST-tagged rCLIC proteins as a model system to test whether the rCLIC proteins can protect bacterial cells from oxidative stress. Such oxidative stress was mimicked *in vitro* by hydrogen peroxide, a known oxidative stress inducer. For this purpose, we compared the growth of *E. coli* BL21 (DE3) bacterial cells transformed to express the rCLIC proteins or empty vector in the presence and absence of H_2O_2 at different concertation of H_2O_2 (2.5,5.0 and 10 mM).

First, we needed to determine working concentrations of hydrogen peroxide where we could challenge the cells without completely killing them. An initial experiment showed that 10.0 mM H_2O_2 resulted in at least a 50% inhibition of the growth of the *E.coli* cells at 3 hours As shown in Figure 3.2, what is also interesting to note, is that the growth rate of cells transformed with the CLIC1-wt appeared to be higher than the cells transformed with the empty vector or CLIC1-Cys24S over the 3 hours of incubation in different concentrations of H_2O_2 (2.5, 5.0 and 10 mM), however, this was not statistically significant at all points. This was seen consistently in all experiments, even when the cells were not challenged with H_2O_2.

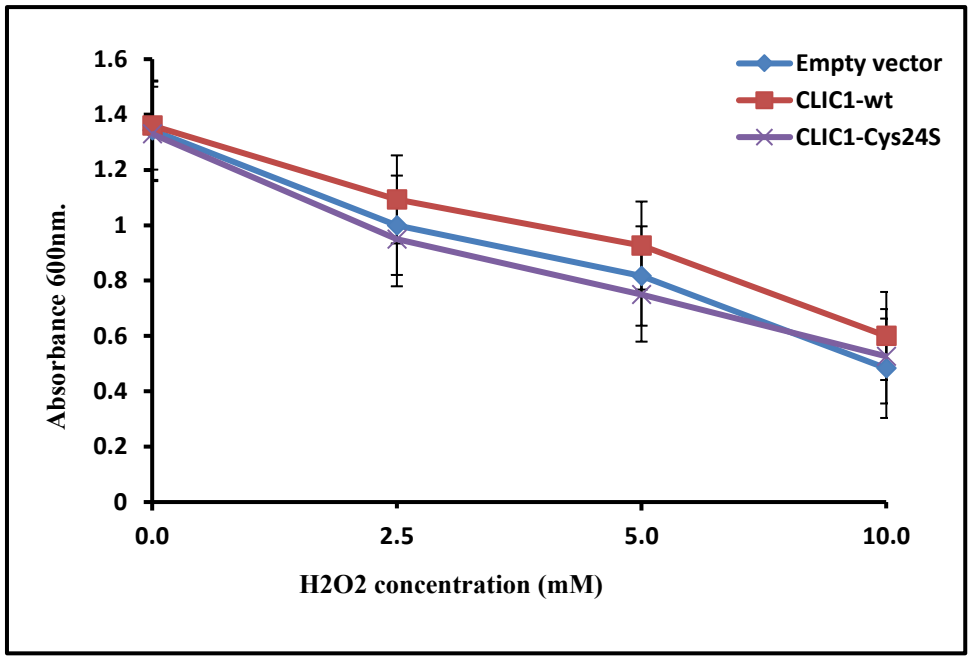

Figure 3. 2 Growth comparison of E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3 hours of incubation in different concentration of H_2O_2 (2.5,5.0 and 10 mM). The results were performed using excel 2010 and the error bars indicate the standard deviation from three independent readings.

Further statistical analysis was carried out using Graph pad prism 8 excel, according to ANOVA statistical test analysis comparing the growth rate for the cells transformed with the CLIC1-wt, the empty vector and the mutant construct CLIC1-Cys24S, grown in the presence or absence of H_2O_2 at different concentrations (2.5,5.0 and 10 mM). As seen in Figure 3.3, showing the absorbance at 600nm for each cell line, the analysis reveals that there is a significant difference in the growth rate between the cells transformed CLIC1-wt when it compared with the mutant CLIC1-Cys24S at the concentration 2.5 mM H_2O_2 as indicated by ordinary one-way ANOVA multiple comparisons test where the p value obtained is ($p< 0.05$). Again, the growth rate for the

cells transformed with the CLIC1-wt was higher than the CLIC1-Cys24S as indicated by ordinary one-way ANOVA multiple comparisons test where the p value obtained is ($p < 0.01$) at 5 mM H_2O_2. Furthermore, the cells transformed with the CLIC1-wt showed a significant difference in the growth rate compared with the cells transformed with the empty vector only at 10 mM H_2O_2 as indicated by ordinary one-way ANOVA multiple comparisons test where the final p value obtained is ($p < 0.01$) Figure 3.3 and Table 3.1, showed the p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

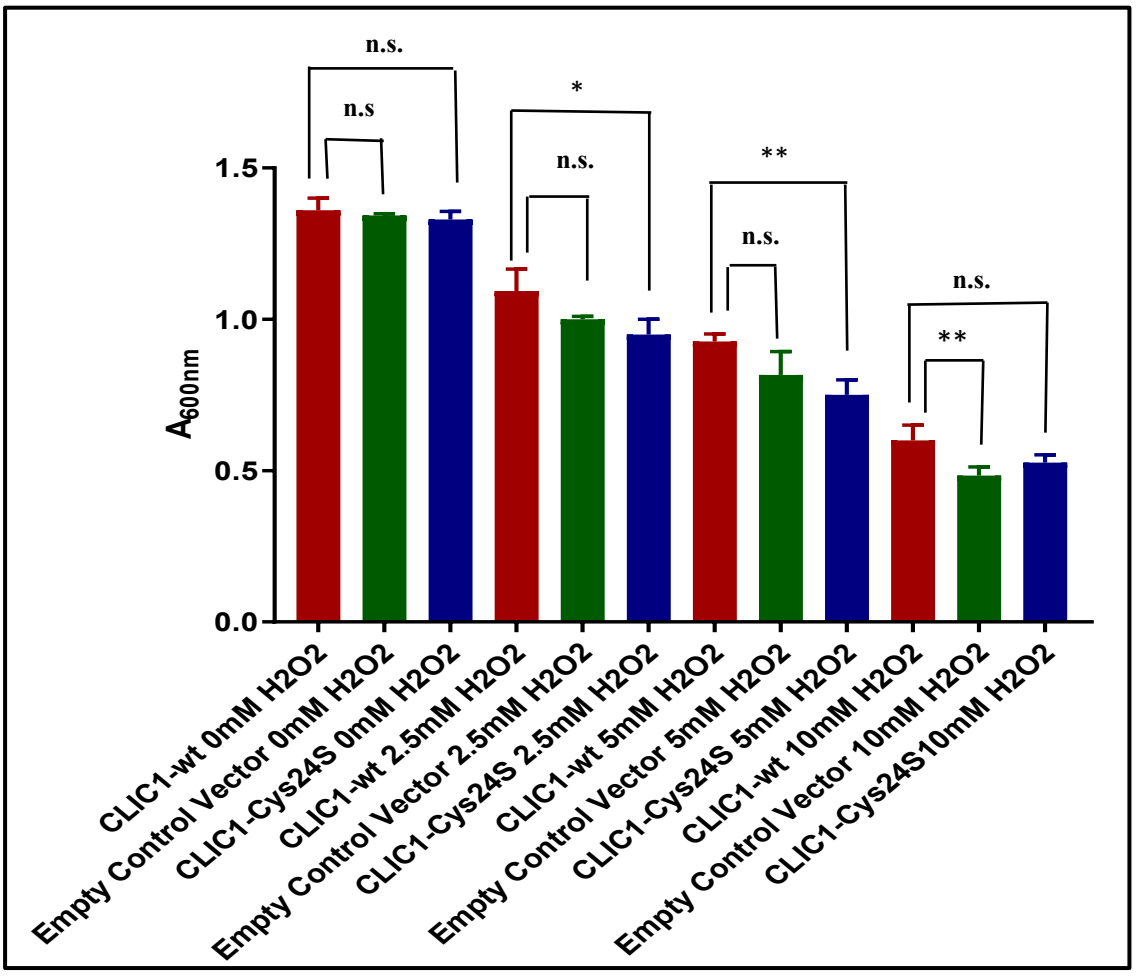

Figure 3. 3 Growth comparison of E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys 24S in pET28a plasmids

after 3 hours of incubation in different concentration of H_2O_2 (2.5,5.0 and 10 mM). The analysis was performed using Graph pad prism 8. Data shown are means ± S.E of at least three independent experiments. The asterisks *and ** correspond to significant (p< 0.05 and p<0.01 respectively) difference in the growth rate of the cells (N=3).

Table 3. 1 Test results comparing the growth rate of the E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3 hours of incubation started at 0.6 OD in the presence and absence of H_2O_2 at different concentration (2.5,5.0 and 10 mM). The growth rate was monitored over 3 hours of incubation. p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

Bacterial cells	Difference in activity	P Value
CLIC1-wt 0mM H2O2 vs. Empty Control Vector 0mM H2O2	Non- significant	>0.9999
CLIC1-wt 0mM H2O2 vs. CLIC1-Cys24S 0mM H2O2	Non- significant	0.9999
CLIC1-wt 2.5mM H2O2 Column vs. Empty Control Vector 2.5mM H2O2	Non-significant	0.3280
CLIC1-wt 2.5mM H2O2 vs. CLIC1-Cys24S 2.5mM H2O2	significant	0.0124
CLIC1-wt 5mM H2O2 vs. Empty Control Vector 5mM H2O2	Non-significant	0.7003

CLIC1-wt 5mM H2O2 vs. CLIC1-Cys24S 5mM H2O2	significant	0.0008
CLIC1-wt 10mM H2O2 vs. Empty Control Vector 10mM H2O2	significant	<0.0001
CLIC1-wt 10mM H2O2 vs. CLIC1-Cys24S10mM H2O2	Non-significant	0.7003

The effect of the oxidant H_2O_2 (10mM) was further assessed on the growth of *E. coli* bacterial cells transformed to express the CLIC1-wt protein, cells transformed with pET28a only or the mutant CLIC1-Cys24S, over a more prolonged exposure time. As seen in Figure 3.4, using 10.0 mM concentration of H_2O_2, the growth of *E. coli* cells transformed with the empty vector, CLIC1-wt and CLIC1-Cys24S was radically reduced following addition of the H_2O_2 at 150 minutes, then left to grow for a total of 6 hours. This finding led us to conclude that 10.0 mM concentration of H_2O_2 was an inhibitory concentration for the growth of the *E.coli* bacterial cells over the time period that we wished to run subsequent experiments. Therefore, a. lower concentration of 5.0 mM H_2O_2 was used instead.

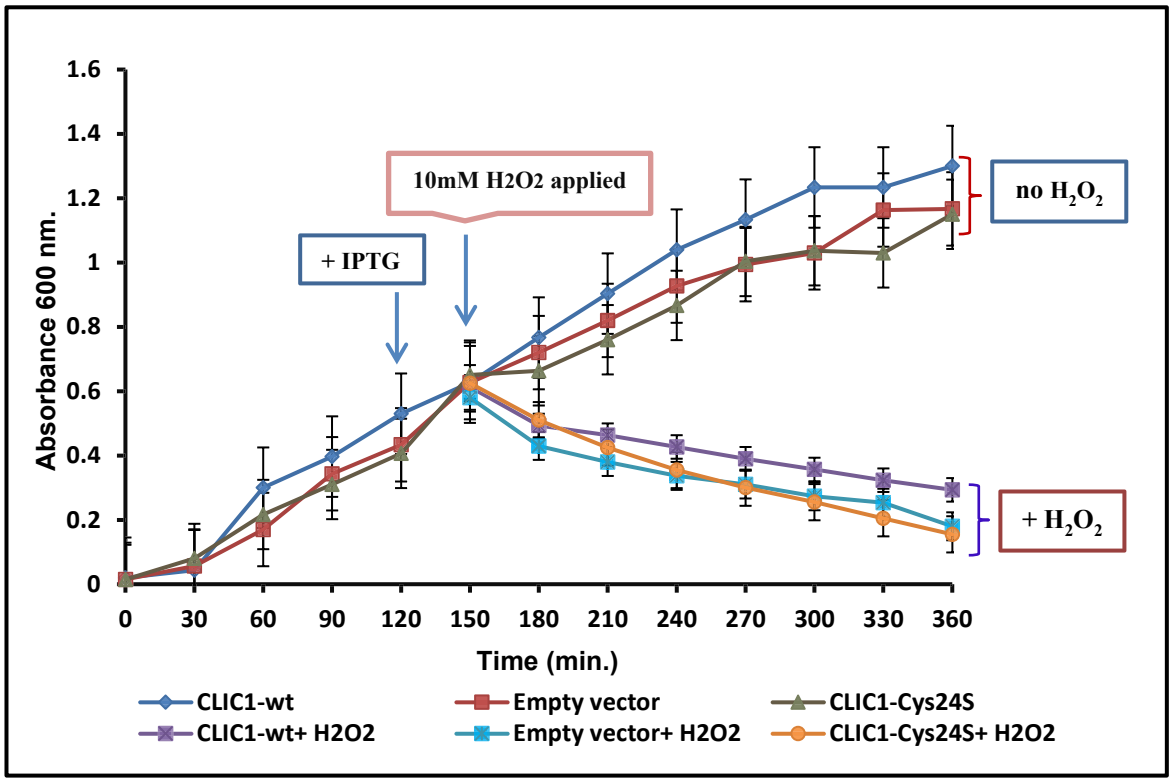

Figure 3. 4 Growth of E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 10 mM. H_2O_2. The growth rate was monitored over 6 hours of incubation. The results were performed using excel 2010 and the error bars indicate the standard deviation from three independent readings.

Statistical analysis was carried out using Graph pad prism 8 excel, according to ordinary one-way ANOVA statistical multiple comparisons test analysis we compared the growth rate for the cells transformed with either the CLIC1-wt protein, the cells transformed with the empty vector or CLIC1-Cys24S in the absence and presence of 10 mM. H_2O_2. Figure 3.5 shows the absorbance at 600nm for each cell line, The CLIC1-wt overexpressing cells show a significant increase in their growth rate when compared to

the cells transformed with pET28a only (p= 0.0280) in the presence of 10 mM of H_2O_2. This further supports the proposal that the higher concentration of 10 mM H_2O_2 in our experiment led to cell death.

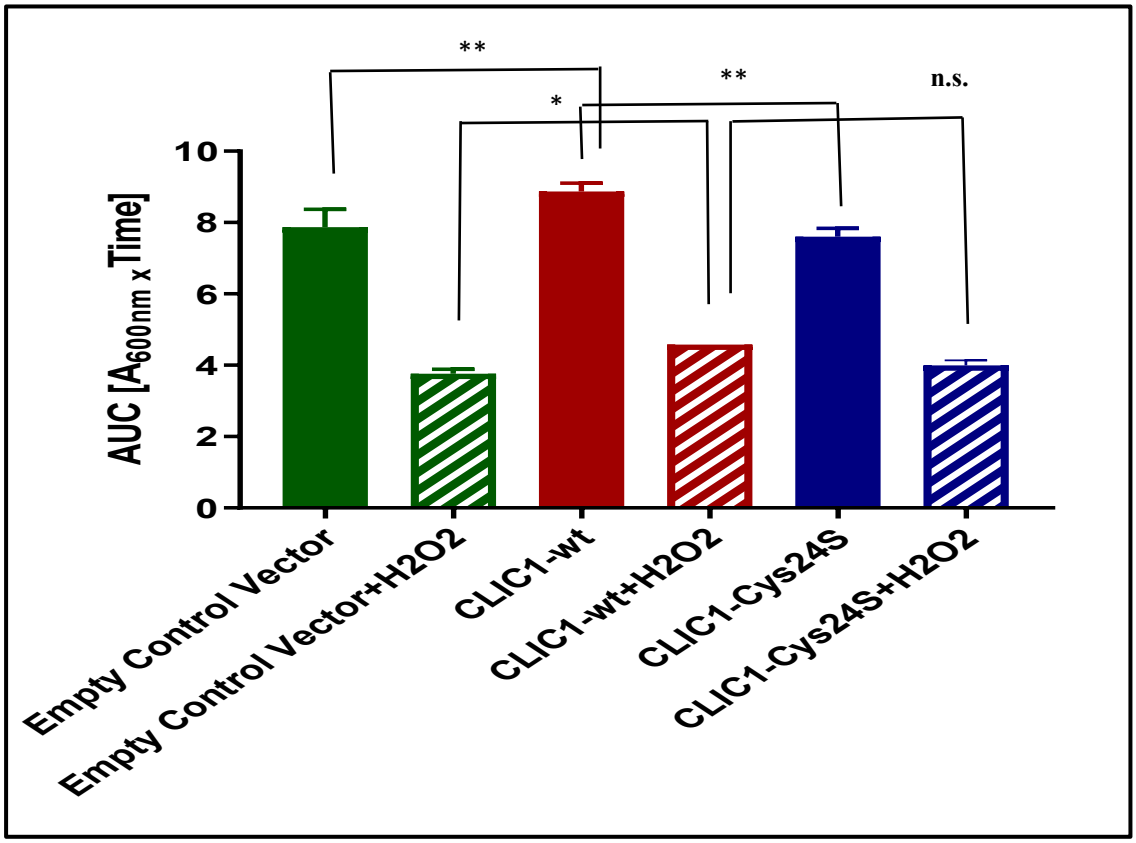

Figure 3. 5 Growth of E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 10 mM. H_2O_2. The growth rate was monitored over 6 hours of incubation. The analysis was performed using Graph pad prism 8. Data shown are means ± S.E of at least three independent experiments. The asterisks *and ** correspond to significant ($p<0.05$ and $p<0.01$ respectively) difference in the growth rate of the cells (N=3).

Moreover, further statistical analysis was performed by carrying out the ordinary one-way ANOVA multiple comparisons statistical test (results as shown in table 3.2).

Bacterial cells	Difference in activity	P Value
CLIC1-wt vs. Empty Control Vector	Significant	0.0077
CLIC1-wt vs. CLIC1-Cys24S	Significant	0.0011
CLIC1-wt vs. CLIC1-wt+H2O2	Significant	<0.0001
CLIC1-wt vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
CLIC1-wt vs. CLIC1-Cys24S+ H_2O_2	Significant	<0.0001
Empty Control Vector vs. CLIC1-Cys24S	Non-significant	0.8239
Empty Control Vector vs. CLIC1-wt+ H_2O_2	Significant	<0.0001
Empty Control Vector vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
Empty Control Vector vs. CLIC1-Cys24S+ H_2O_2	Significant	<0.0001
CLIC1-Cys24S vs. CLIC1-wt+ H_2O_2	Significant	<0.0001
CLIC1-Cys24S vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
CLIC1-Cys24S vs. CLIC1-Cys24S+ H_2O_2	Significant	<0.0001
CLIC1-wt+ H_2O_2vs. Empty Control Vector+ H_2O_2	Significant	0.0280
CLIC1-wt+ H_2O_2 vs. CLIC1-Cys24S+ H_2O_2	Non-significant	0.1614
Empty Control Vector+ H_2O_2 vs. CLIC1-Cys24S+ H_2O_2	Non-significant	0.8854

Table 3. 2 Test results comparing the growth rate of the E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3 hours of incubation started at 0.6 OD in the

absence and presence of 10 mM. H2O2. The growth rate was monitored over 6 hours of incubation. p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

Thus, we investigated the growth rate of the *E.coli* bacterial cells in the presence of 5.0 mM H_2O_2 over 6 hours In this experiment, the 3 different bacterial cell lines (empty vector controls, CLIC1-wt overexpressing cells and a CLIC1-Cys24S mutant) were grown and induced with IPTG at 2 hours then challenged with 5.0 mM H_2O_2 at 150 minutes, their growth rate was monitored over 6 hours. As seen in Figure 3.6, CLIC1-wt overexpressing cells grew better than the cells transformed with pET28a only or mutant CLIC1-Cys24S in pET28a plasmid after additional of 5.0 mM H_2O_2 over 6 hours.

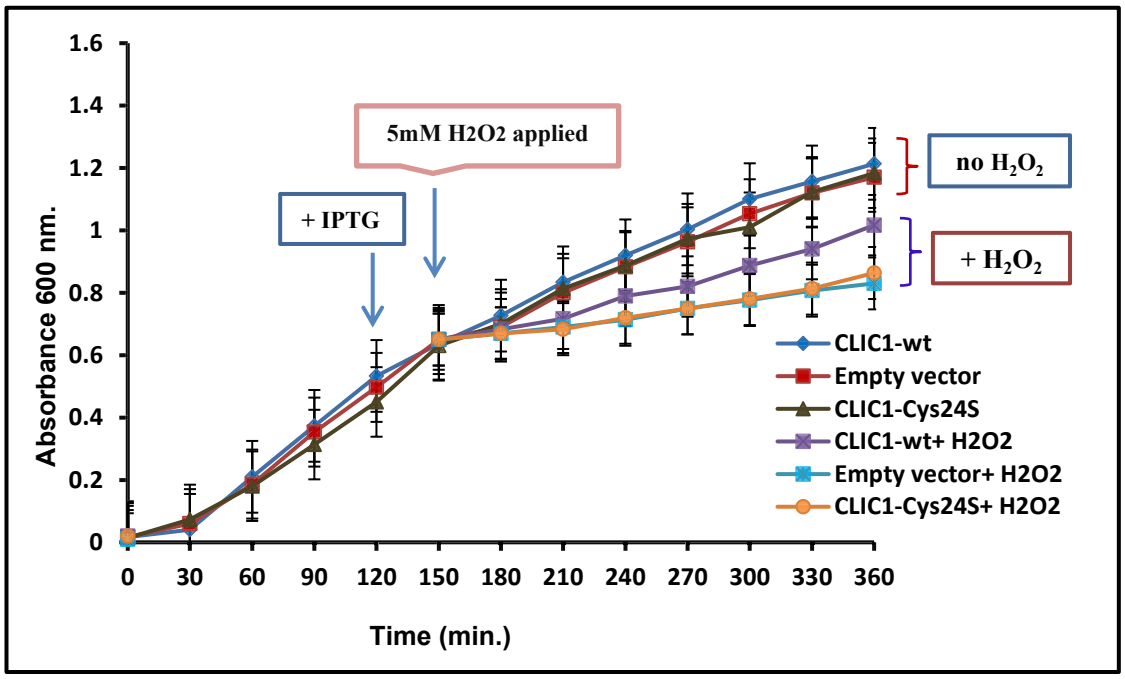

Figure 3. 6 Growth of E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5 mM H2O2.

The growth rate was monitored over 6 hours of incubation. The results were performed using excel 2010 and the error bars indicate the standard deviation from three independent readings.

Furthermore, as seen in Figure 3.7 reveals the absorbance at 600nm for each cell line, using ordinary one-way ANOVA statistical multiple comparisons test analysis, CLIC1-wt overexpressing cells present a significantly higher growth rate compared to the cells transformed with pET28a only in the presence of 5 mM H_2O_2 as the ($p = 0.0070$) or CLIC1-Cys24S in pET28a plasmid where the ($p = 0.01430$). This result supports our hypothesis that the CLIC1 wild-type protein was providing some level of antioxidant cell protective effect.

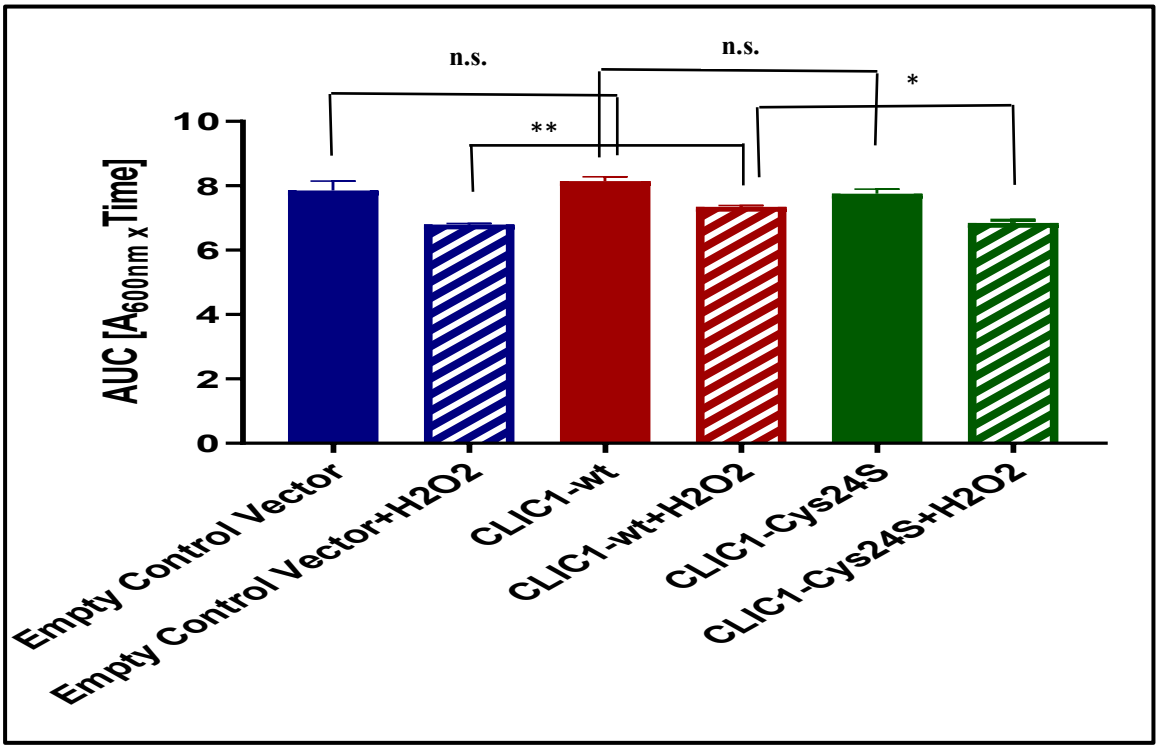

Figure 3. 7 Growth of E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3

hours of incubation started at 0.6 OD in the absence and presence of 5 mM H_2O_2. The growth rate was monitored over 6 hours of incubation. The analysis was performed using Graph pad prism 8. The error bars represent the standard error of three independent repeats of growth rate measurements. (N=3). Data shown are means ± S.E of at least three independent experiments. The asterisks * and ** correspond to significant (p<0.05 and p<0.01 respectively) difference in the growth rate of the cells (N=3).

Moreover, further statistical analysis was performed by carrying out the ordinary one-way ANOVA multiple comparisons statistical test (results as shown in table 3.3).

Bacterial cells	**Difference in activity**	**P Value**
CLIC1-wt vs. Empty Control Vector	Non-significant	0.2105
CLIC1-wt vs. CLIC1-Cys24S	Non-significant	0.0578
CLIC1-wt vs. CLIC1-wt+ H_2O_2	Significant	0.0003
CLIC1-wt vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
CLIC1-wt vs. CLIC1-Cys24S+ H_2O_2	Significant	<0.0001
Empty Control Vector vs. CLIC1-Cys24S	Non-significant	0.9597
Empty Control Vector vs. CLIC1-wt+ H_2O_2	Significant	0.0127
Empty Control Vector vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
Empty Control Vector vs. CLIC1-Cys24S+ H_2O_2	Significant	<0.0001
CLIC1-Cys24S vs. CLIC1-wt+ H_2O_2	Significant	0.0499
CLIC1-Cys24S vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
CLIC1-Cys24S vs. CLIC1-Cys24S+ H_2O_2	Significant	<0.0001

CLIC1-wt+ H_2O_2 vs. Empty Control Vector+ H_2O_2	Significant	0.0070
CLIC1-wt+ H_2O_2vs. CLIC1-Cys24S+ H_2O_2	Significant	0.0143
Empty Control Vector+ H_2O_2vs. CLIC1-Cys24A+ H_2O_2	Non-significant	0.9977

Table 3. 3 Test results comparing the growth rate of the E. coli BL21 (DE3) cells transformed with pET28a (empty vector), wild-type CLIC1 in pET28a and CLIC1-Cys24S in pET28a plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5 mM. H_2O_2. The growth rate was monitored over 6 hours of incubation. p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

Furthermore, in order to confirm the critical role of the Cysteine residue located in the CLIC1 enzyme active site, the mutant version CLIC1-Cys24S was assayed at different concentrations of H_2O_2 (2.5, 5.0 and 10.0 mM). Results showed that the growth rate of the E. *coli* BL21 (DE3) CLIC1-Cys24S mutant cells significantly lower than the E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC1-wt protein as seen in Figure 3.3.

Moreover, the growth rate of *E. coli* bacterial cells transformed to express the mutant CLIC1-Cys24S was analysed over 6 hours in the presence of 5 mM H_2O_2. Results in Figure 3.6 showed that the growth rate of the mutant CLIC1-Cys24S cells dropped dramatically when it compared to the E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC1-wt protein as the P value was (p= 0.0143) in the presence of 5 mM. of the H_2O_2 and the growth rate was monitored over 6 hours of incubation as seen in Figure 3.7. This confirms that the single active site cysteine in CLIC1 protein (Cys-24) is essential for the cellular antioxidant activity of the CLIC1 proteins.

3. 6 Assessment of CLIC3 cellular antioxidant activity

E. coli BL21 (DE3) bacterial cells transformed to express the recombinant wild type CLIC3 protein were used to determine the intracellular antioxidant activity CLIC3. In this experiment, the *E. coli* BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector as control), wild type CLIC3 in pGEX-6P-1 and the mutant form of CLIC3 - where the critical dithiols in the enzyme active site were mutated to alanine - CLIC3-Cys 22,25A in pGEX-6P-1 plasmids, were exposed to different concentrations of H2O2 (2.5, 5.0, 10.0 mM). As you can see in Figure 3.8, the growth rate of the CLIC3 wild type overexpressing cells, like *E. coli* BL21 (DE3) cells transformed with wild-type CLIC1 in pET28a appear to grow better than the cells transformed with the vector only or the mutant CLIC3-Cys 22,25A in pGEX-6P-1 plasmid in the presence of 2.5 mM concentrations of H2O2 over a 3 hours period. Furthermore, the three group of cells tested in this experiment showed similar growth rate in the presence of 5.0 mM of H2O2 over a 3 hours period of incubation. In addition, the growth rate of the mutant CLIC3-Cys 22,25A in pGEX-6P-1 plasmid appear to grow better than the cells transformed with the vector only or the CLIC3 wild type overexpressing cells in the presence of 10.0 mM of H2O2 over a 3 hours period of incubation.

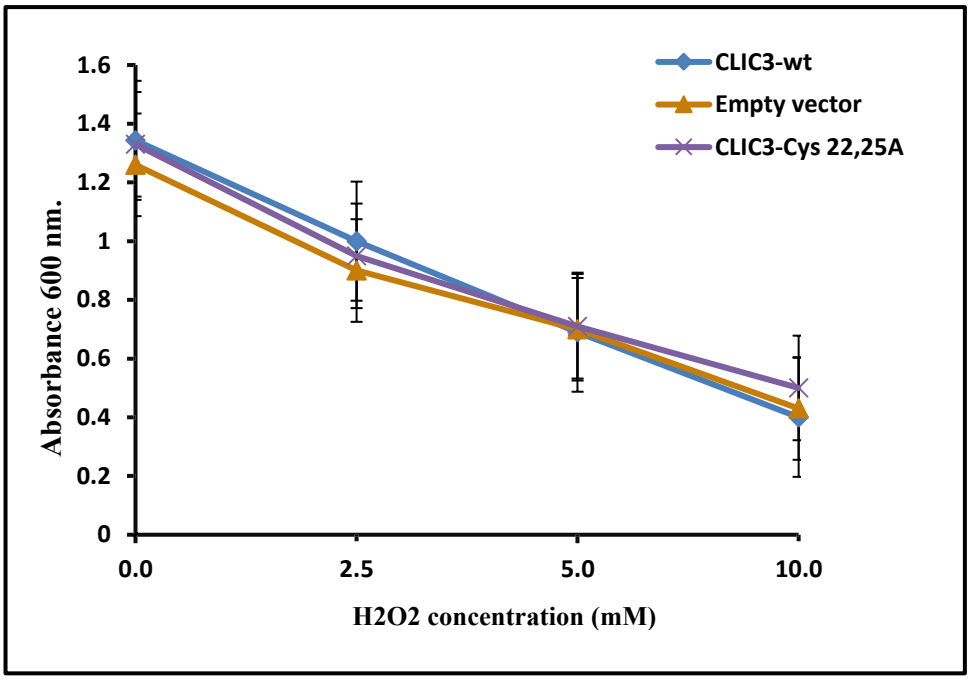

Figure 3. 8 Growth of E. coli BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild type CLIC3 in pGEX-6P-1 and CLIC3-Cys 22,25A in pGEX-6P-1 plasmids after 3 hours of incubation in different concentration of H_2O_2 (2.5,5.0 and 10 mM). The results were performed using excel 2010 and the error bars indicate the standard deviation from three independent readings.

Following analysis of the data by comparing the absorbance at 600nm for each cell line, a significant difference in the growth rate using ordinary one-way ANOVA multiple comparison statistical test analysis was determined. It was found that the CLIC3-wt overexpressing cells did not showed any significant difference in their growth rate when compared to the cells transformed with pGEX-6P-1 only or CLIC3-Cys 22,25A in pGEX-6P-1 plasmid after 3 hours of incubation in different concentration of H_2O_2 (2.5,5.0 and 10 mM), Figure 3.9 and Table 3.4, showed the p value obtained by performing ordinary one-way ANOVA statistical test analysis. Although not statistically

significant, but there is a trend seen were the CLIC3-wt overexpressing cells do appear to grow better than the cells transformed with pGEX-6P-1 only or CLIC3-Cys 22,25A in pGEX-6P-1 plasmid after 3 hours of incubation in different concentration of H_2O_2 (2.5,5.0 and 10 mM), Figure 3.8

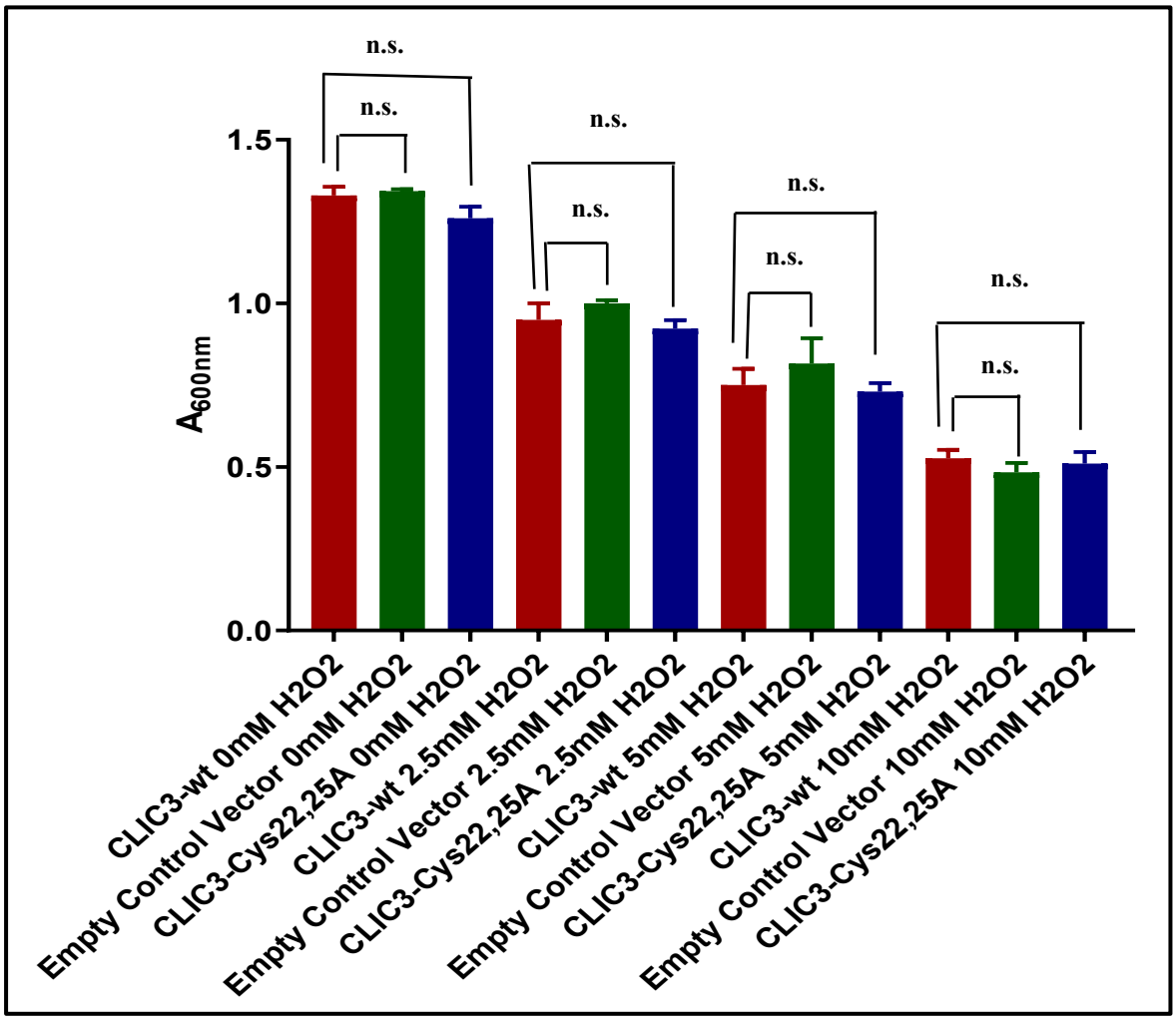

Figure 3. 9 Growth of E. coli BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild type CLIC3 in pGEX-6P-1 and CLIC3-Cys 22,25A in pGEX-6P-1 plasmids after 3 hours of incubation in different concentration of H_2O_2 (2.5,5.0 and 10 mM). The analysis was performed using Graph pad prism 8. Data shown are means ± S.E of at least three independent experiments. The asterisks * and *

correspond to significant (p<0.05 and p<0.01 respectively) difference in the growth rate of the cells (N=3).

Table 3. 4 Test results comparing the growth rate of the E. coli BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild-type CLIC3 in pGEX-6P-1 and CLIC3-Cys22,25A in pGEX-6P-1 plasmids after 3 hrs. of incubation started at 0.6 OD in the presence and absence of H_2O_2 at different concentration (2.5,5.0 and 10 mM). The growth rate was monitored over 3 hours of incubation. p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

Bacterial cells	**Difference in activity**	**P Value**
CLIC3-wt 0mM H_2O_2 vs. Empty Control Vector 0mM H_2O_2	Non- significant	>0.9999
CLIC3-wt 0mM H_2O_2 vs. CLIC3-Cys22,25A 0mM H_2O_2	Non- significant	0.7605
CLIC3-wt 2.5mM H_2O_2 vs. CLIC3-Cys22,25A 2.5mM H_2O_2	Non-significant	>0.9999
CLIC3-wt 2.5mM H_2O_2 vs. CLIC3-Cys22,25A 2.5mM H_2O_2	Non-significant	>0.9999
CLIC3-wt 5mM H_2O_2 vs. Empty Control Vector 5mM H_2O_2	Non-significant	0.8153
CLIC3-wt 5mM H_2O_2 vs. CLIC3-Cys22,25A 5mM H_2O_2	Non-significant	>0.9999
CLIC3-wt 10mM H_2O_2 vs. Empty Control Vector 10mM H_2O_2	Non-significant	0.9935

CLIC3 wild type overexpressing cells, cells transformed with the vector only and cells transformed the mutant CLIC3-Cys22,25A in pGEX-6P-1 plasmids were further assessed to determine the intracellular antioxidant activity of the CLIC3 proteins in the presence of 5.0 mM H_2O_2 over a 6 hours period. As seen in Figure 3.10, CLIC3-wt overexpressing cells appear to grow better than the empty vector and CLIC3-Cys22,25A mutant cell lines.

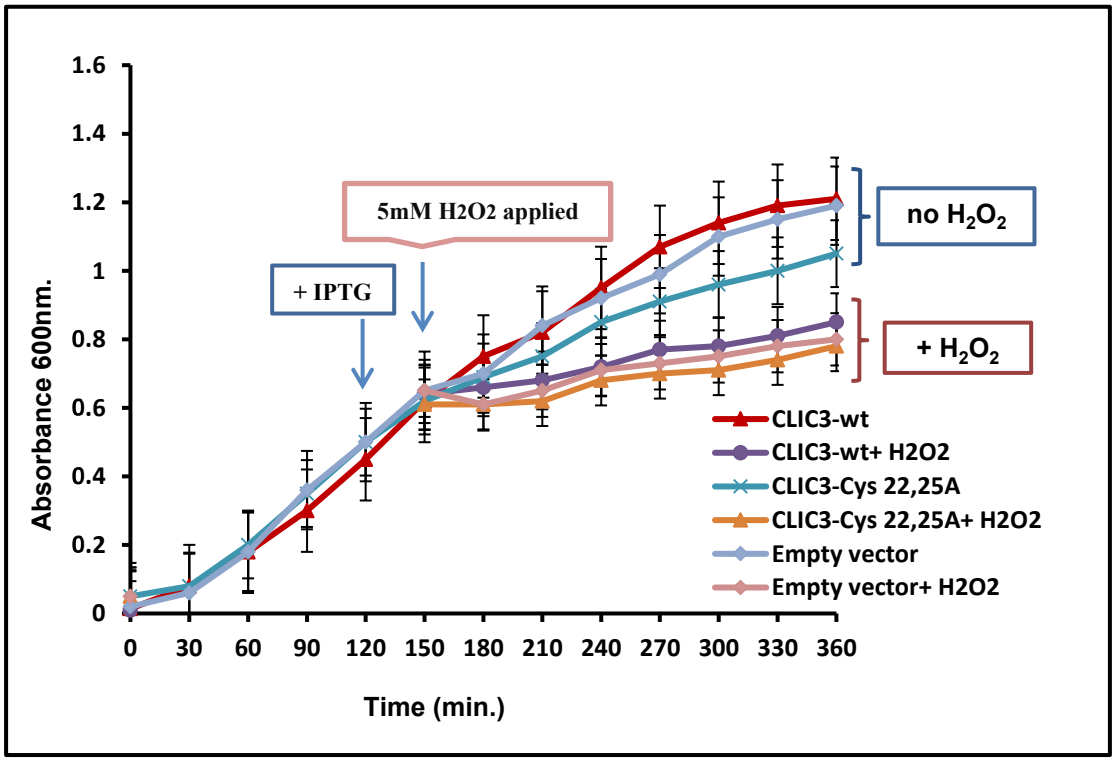

Figure 3. 10 Growth of* E. coli *BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild type CLIC3 in pGEX-6P-1 and CLIC3-Cys 22,25A in pGEX-6P-1 plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5 mM. H_2O_2 over increasing period. The growth rate was monitored over 6 hours of incubation. The results were performed using excel 2010 and the error bars indicate the standard deviation from three independent readings.

Additionally, as seen in Figure 3.11 using ordinary one-way ANOVA multiple comparison statistical test analysis of the absorbance at 600nm calculated for each cell line, indicated that the CLIC3-wt overexpressing cells did not showed any significantly difference in the growth rate when compared to the cells transformed with pGEX-6P-1 only in the presence of 5 mM. H_2O_2 as the P value obtained is ($p = 0.0582$). Although not statistically significant, but there is a trend seen were the CLIC3-wt overexpressing cells do appear to grow better than the cells transformed with pGEX-6P-1 only in the presence of 5 mM. H_2O_2, Figure 3.10. However, results showed that there is a higher significant difference in the growth rate of the CLIC3-wt overexpressing cells when it compared to the mutant CLIC3-Cys22,25A in pGEX-6P-1 plasmids as the P value is ($p =0.0024$). This result also supports the hypothesis that wild type CLIC3 protein provides some antioxidant cell protective effect.

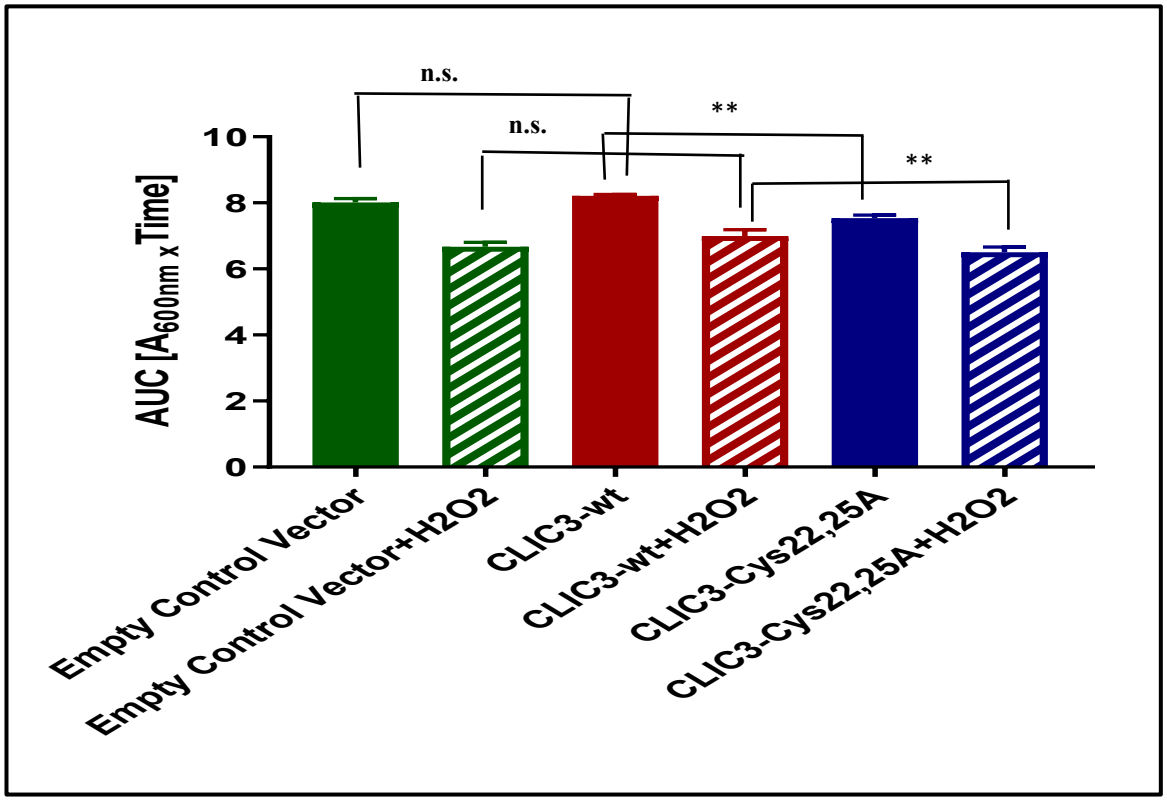

Figure 3. 11 Growth of E. coli BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild type CLIC3 in pGEX-6P-1 and CLIC3-Cys 22,25A in pGEX-6P-1 plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5 mM. H_2O_2 over increasing period. The growth rate was monitored over 6 hours of incubation. The analysis was performed using Graph pad prism 8. Data shown are means ± S.E of at least three independent experiments. The asterisks * and ** correspond to significant ($p<0.05$ and $p<0.01$ respectively) difference in the growth rate of the cells (N=3).

Moreover, further statistical analysis was performed by carrying out the ordinary one-way ANOVA multiple comparisons statistical test (results as shown in table 3.5).

Bacterial cells	**Difference in activity**	**P Value**
CLIC3-wt vs. CLIC3-wt+ H_2O_2	Significant	<0.0001
CLIC3-wt vs. CLIC3-Cys22,25A	Significant	<0.0001
CLIC3-wt vs. CLIC3-Cys22,25A+ H_2O_2	Significant	<0.0001
CLIC3-wt vs. Empty Control Vector	Non-significant	0.5274
CLIC3-wt vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
CLIC3-wt+ H_2O_2 vs. CLIC3-Cys22,25A	Significant	0.0010
CLIC3-wt+ H_2O_2 vs. CLIC3-Cys22,25A+ H_2O_2	Significant	0.0024
CLIC3-wt+ H_2O_2 vs. Empty Control Vector	Significant	<0.0001
CLIC3-wt+ H_2O_2 vs. Empty Control Vector+ H_2O_2	Non-significant	0.0582
CLIC3-Cys22,25A vs. CLIC3-Cys22,25A+ H_2O_2	Significant	<0.0001
CLIC3-Cys22,25A vs. Empty Control Vector	Significant	0.0031

CLIC3-Cys22,25A vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
CLIC3-Cys22,25A+ H_2O_2 vs. Empty Control Vector	Significant	<0.0001
CLIC3-Cys22,25A+ H_2O_2 vs. Empty Control Vector+ H_2O_2	Non-significant	0.7174
Empty Control Vector vs. Empty Control Vector+ H_2O_2	Significant	<0.0001

Table 3. 5 Test results comparing the growth rate of the E. coli BL21 (DE3) cells transformed with pGEX-6P-1 (empty vector), wild type CLIC3 in pGEX-6P-1 and CLIC3-Cys 22,25A in pGEX-6P-1 plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5 mM. H_2O_2. The growth rate was monitored over 6 hours of incubation. p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

Additionally, in order to determine the critical role of the Cysteine residues located in the CLIC3 enzyme active site, the mutant version CLIC3-Cys22,25A was exposed to different concentrations of H_2O_2 (2.5, 5.0 and 10.0 mM). Results showed that the growth rate of the E. *coli* BL21 (DE3) rCLIC1-Cys22,25A mutant cells were significantly lower than the E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC3-wt protein as seen in Figure 3.8. Furthermore, the bacterial cells transformed to express the CLIC3-wt protein cells did not showed any significant difference in their growth rate when compared to the cells transformed with pGEX-6P-1 only as the P value was ($p = 0.5182$) or to the cells CLIC3-Cys 22,25A in pGEX-6P-1 plasmid ($p = 0.1029$) as seen in Figure 3.9. Although not statistically significant, but there is a trend seen were the CLIC3-wt overexpressing cells do appear to grow better than the cells transformed with pGEX-6P-1 only or to the cells CLIC3-Cys 22,25A in pGEX-6P-1

plasmid in the presence of different concentrations of H2O2 (2.5, 5.0 and 10.0 mM). H2O2, Figure 3.10.

Furthermore, the growth rate of *E. coli* bacterial cells transformed to express the mutant CLIC3-Cys22,25A was monitored over 6 hours in the presence of 5.0 mM H_2O_2. Results in Figure 3.10 revealed that the growth rate of the mutant CLIC3-Cys22,25A cells significantly decreased when it compared to the E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC3-wt protein. Results in Figure 3.11 showed that the growth rate of the mutant CLIC3-Cys22,25A cells dropped when it compared to the E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC3-wt protein as the P value was (P= 0.0024) in the presence of 5 mM . Unlike CLIC1, CLIC3 protein contain dithiol active site cysteines (Cys-22 and Cys-25). Interestingly, our results provide a strong indicative that both Cysteines (Cys-22 and Cys-25) are essential for the cellular antioxidant activity of the CLIC3 proteins.

3. 7 Assessment of CLIC4 cellular antioxidant activity

CLIC4-wt was also assessed for potential antioxidant protective functions. The growth rate of *E. coli* BL21 (DE3) bacterial cells transformed to express the recombinant wild type CLIC4 protein was compared to vector control transformed cells. As seen in Figure 3.12, the growth rate of E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC4-wt protein appears to be marginally higher than the cells transformed with pGEX-2T vector, across a range of different H_2O_2 (2.5, 5.0, 10.0 mM) concentrations.

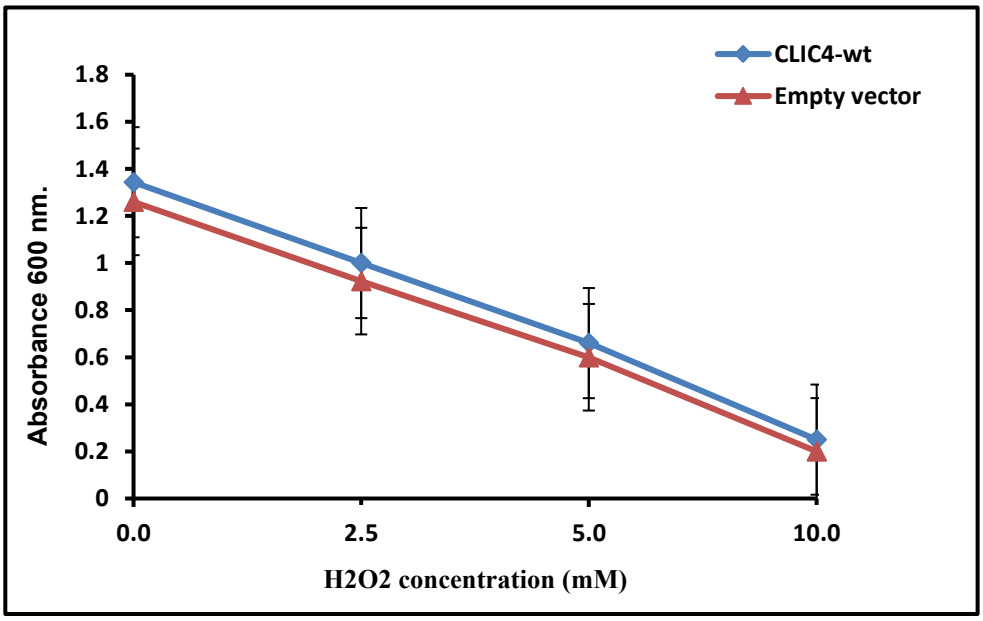

Figure 3. 12 Growth of E. coli BL21 (DE3) cells transformed with pGEX-2T (empty vector), and wild-type CLIC4 in pGEX-2T plasmids after 3 hours of incubation in different concentration of H2O2 (2.5,5.0 and 10 mM). The results were performed using excel 2010 and the error bars indicate the standard deviation from three independent readings.

Figure 3.13 shows the absorbance at 600nm for each cell line, from graph in Figure 3.12. However, the growth rate of *E. coli* BL21 (DE3) bacterial cells transformed to express the CLIC4-wt protein appears to be marginally higher than the cells transformed with pGEX-2T vector, across a range of different H_2O_2 (2.5, 5.0, 10.0 mM) concentrations. However, the statistical analysis using ordinary one-way ANOVA multiple comparison statistical test analysis, demonstrates that the CLIC4-wt overexpressing cells did not show any significant difference in their growth rate, compared to the cells transformed with pGEX-2T only, as shown in Table 3.6, revealed the p values obtained by performing ordinary one-way ANOVA multiple comparison

statistical analysis test. Although not statistically significant, but there is a trend seen were the CLIC4-wt overexpressing cells do appear to grow better than the cells transformed with pGEX-2T only plasmid in the presence of different concentrations of H2O2 (2.5, 5.0 and 10.0 mM). H2O2, Figure 3.12.

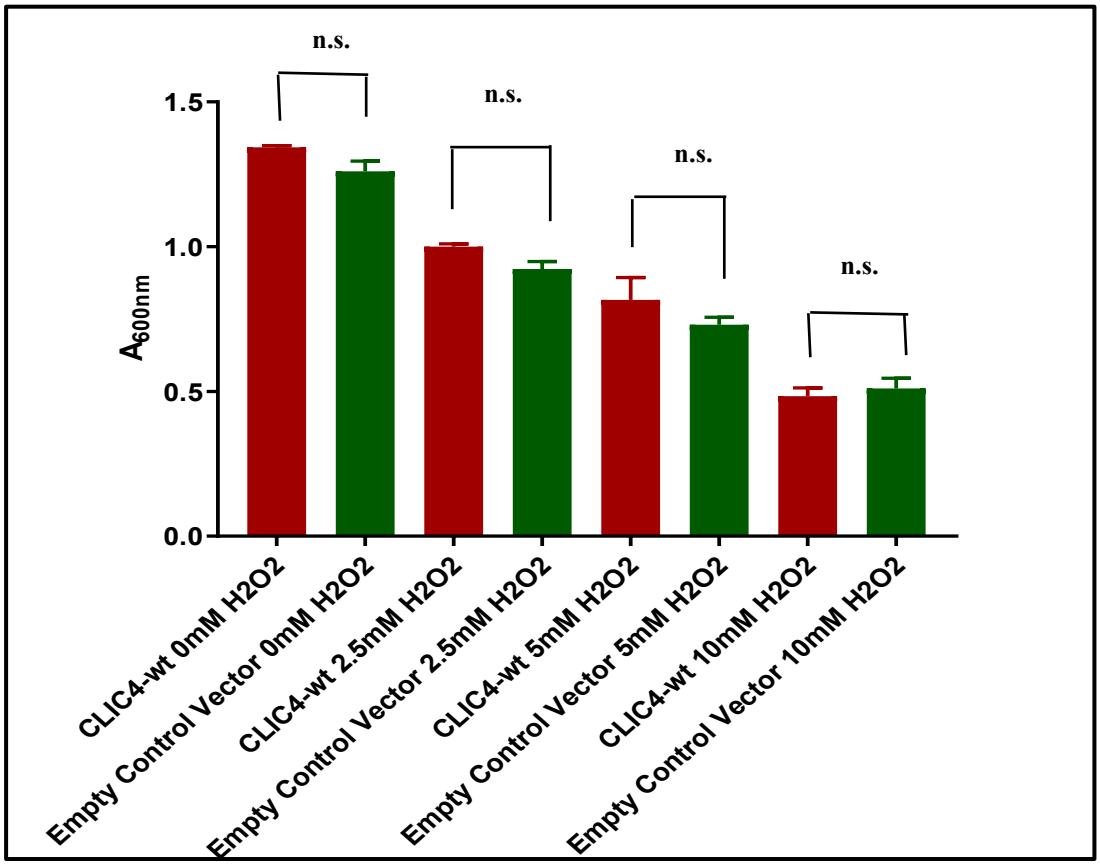

Figure 3. 13 Growth of E. coli BL21 (DE3) cells transformed with pGEX-2T (empty vector), and wild-type CLIC4 in pGEX-2T plasmids after 3 hours of incubation in different concentration of H_2O_2 (2.5,5.0 and 10 mM). The analysis was performed using Graph pad prism 8. Data shown are means ± S.E of at least three independent repeats of growth rate experiments (N=3). The asterisks * and ** correspond to

significant ($p<0.05$ and $p<0.01$ respectively) difference in the growth rate of the cells (N=3).

Table 3. 6 Test results comparing the growth rate of the E. coli BL21 (DE3) cells transformed with pGEX-2T (empty vector), wild-type CLIC4 in pGEX-2T plasmids after 3 hrs. of incubation started at 0.6 OD in the presence and absence of H_2O_2 at different concentration (2.5,5.0 and 10 mM). The growth rate was monitored over 3 hours of incubation. p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

Bacterial cells	**Difference in activity**	**P Value**
CLIC4-wt 0mM H_2O_2 vs. Empty Control Vector 0mM H_2O_2	Non- significant	0.4088
CLIC4-wt 2.5mM H_2O_2 vs. Empty Control Vector2.5mM H_2O_2	Non- significant	0.5264
CLIC4-wt 5mM H_2O_2 vs. Empty Control Vector 5mM H_2O_2	Non-significant	0.3550
CLIC4-wt 10mM H_2O_2 vs. Empty Control Vector 10mM H_2O_2	Non-significant	0.9995

The results in Figure 3.14, showed that the CLIC4-wt overexpressing cells grew the same or only better than the cells transformed with the vector only in the presence of 5.0 mM H_2O_2 over the 6 hours of investigation.

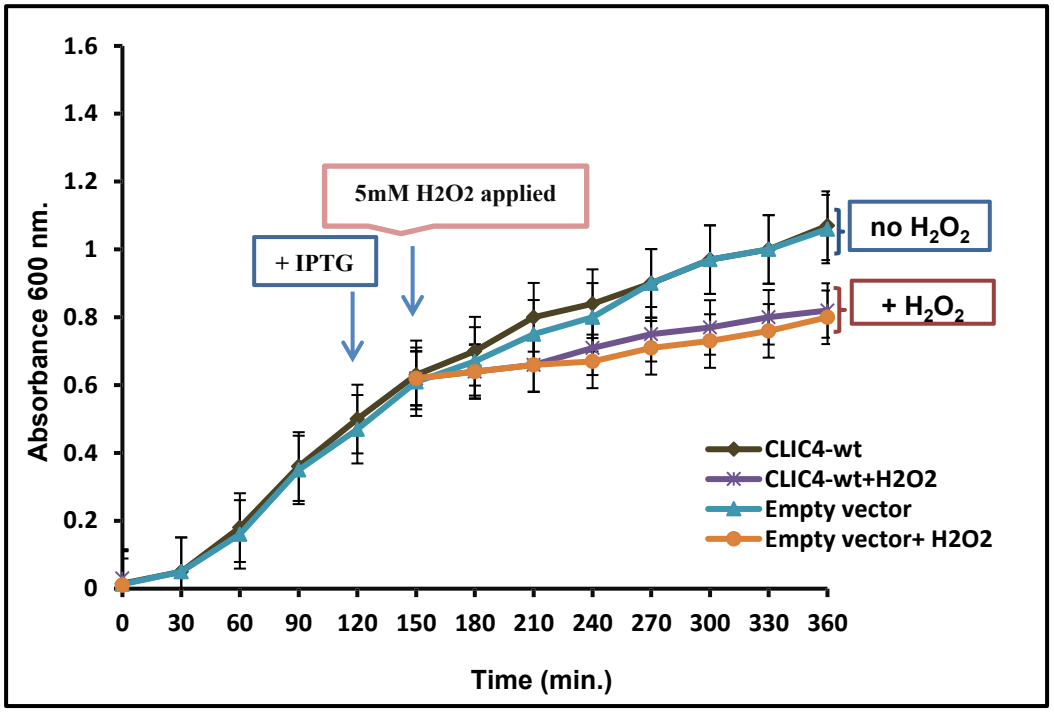

Figure 3. 14 Growth of* E. coli *BL21 (DE3) cells transformed with pGEX-2T (empty vector), and wild type CLIC4 in pGEX-2T plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5.0 mM. H_2O_2 over increasing period. The growth rate was monitored over 6 hours of incubation. The results were performed using excel 2010 and the error bars indicate the standard deviation from three independent readings.

Additionally, as seen in Figure 3.15, shows the area under the curve for each cell line, we determine the P value using ordinary one-way ANOVA multiple comparison statistical test analysis. Even though the CLIC4-wt overexpressing cells appeared to grow better than the cells transformed with the vector only in the presence of 5.0 mM H_2O_2 within 6 hours of investigation, this did not hold true following statistical analysis. The CLIC4-wt overexpressing cells did not showed any significant difference

in the growth rate when compared to the cells transformed with the vector only in the presence of 5.0 mM H_2O_2 as the p value obtained is (p = 0.7432). Although not statistically significant, but there is a trend seen were the CLIC4-wt overexpressing cells do appear to grow better than the cells transformed with pGEX-2T only plasmid in the presence of 5.0 mM H_2O_2, Figure 3.14.

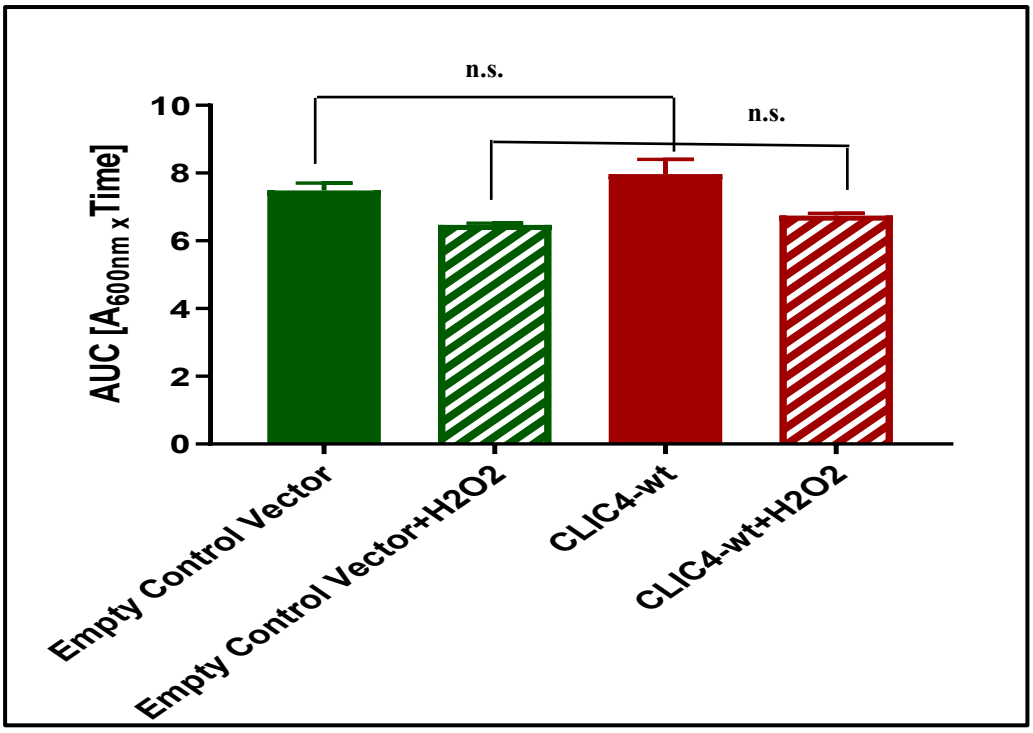

Figure 3. 15 Growth of E. coli BL21 (DE3) cells transformed with pGEX-2T (empty vector), and wild type CLIC4 in pGEX-2T plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5.0 mM. H_2O_2 over increasing period. The growth rate was monitored over 6 hours of incubation. The analysis was performed using Graph pad prism 8. Data shown are means ± S.E of at least three independent repeats of growth rate experiments (N=3). The asterisks * and *

correspond to significant (p<0.05 and p<0.01 respectively) difference in the growth rate of the cells (N=3).

Moreover, further statistical analysis was performed by carrying out the ordinary one-way ANOVA multiple comparisons statistical test (results as shown in table 3.7).

Bacterial cells	**Difference in activity**	**P Value**
CLIC4-wt vs. CLIC4-wt+ H_2O_2	Significant	0.0005
CLIC4-wt vs. Empty Control Vector	Non-significant	0.2120
CLIC4-wt vs. Empty Control Vector+ H_2O_2	Significant	<0.0001
CLIC4-wt+ H_2O_2 vs. Empty Control Vector	Significant	0.0257
CLIC4-wt+ H_2O_2 vs. Empty Control Vector+ H_2O_2	Non-significant	0.7432
Empty Control Vector vs. Empty Control Vector+ H_2O_2	Significant	0.0027

Table 3. 7 Test results comparing the growth rate of the E. coli BL21 (DE3) cells transformed with pGEX-2T (empty vector), and wild type CLIC4 in pGEX-2T plasmids after 3 hours of incubation started at 0.6 OD in the absence and presence of 5 mM. H_2O_2. The growth rate was monitored over 6 hours of incubation. p values obtained by performing ordinary one-way ANOVA test for multiple sample comparisons.

Interestingly, our results showed that of the three rCLICs transformed bacterial cells studied, the cells transformed with rCLIC1 demonstrate highest tolerance to H_2O_2, compared to either rCLIC3 or rCLIC4, when the cells are exposed to different concentrations of H_2O_2 (2.5, 5.0, 10.0 mM) after three hours of incubation. By contrast, statistical analysis showed the area under the curve for each cell line, we determine the p value using ordinary one-way ANOVA multiple comparison statistical test analysis. The cells transformed with rCLIC1-wt showed a significant difference in the growth rate when compared to the growth rate of the CLIC1-Cys24S as the p value obtained was p=0.0124 and p=0.0008 at 2.5 and 5mM concentration of H_2O_2 respectively after three hours of incubation. Furthermore, the cells transformed with rCLIC1-wt revealed a significant difference in the growth rate when it compared to the growth rate of the empty vector at 10mM concentration of H_2O_2 after 3 hours of incubation (result shown in Figure 3.3 and Table 3.1). Furthermore, the results in (Figure 3.11 and Table 3.4), showed that there is a higher significant difference in the growth rate of the CLIC3-wt overexpressing cells when it compared to the mutant CLIC3-Cys22,25A in pGEX-6P-1 plasmids at as the P value is ($p = 0.0024$) at 5mM concentration of H_2O_2 after three hours of incubation. Additionally, the CLIC3-wt overexpressing cells did not showed any significantly difference in the growth rate when compared to the cells transformed with pGEX-6P-1 only in the presence of 5 mM. H_2O_2 as the P value obtained is ($p = 0.0582$) (result shown in Figure 3.11 and Table 3.4).

While, rCLIC4-wt overexpressing cells did not show any significant difference in their growth rate under the same conditions (5mM. H_2O_2), after three hours of incubation., compared to the cells transformed with pGEX-2T only, as shown in Figure 3.13 and

Table 3.6, revealed the p value obtained by performing ordinary one-way ANOVA multiple comparison statistical analysis test is (P= 0.3550).

3. 8 Discussion

ROS are very toxic to organisms, where increased generation of ROS can diminish cellular redox homeostasis leading to post translational modification or/and disruption to the normal function of proteins, DNA and lipid molecules (53, 54). The damage caused by ROS are referred as oxidative stress and are due to excessive levels of ROS. Therefore, ROS attack many biomolecules such as lipids, nucleic acids, proteins and carbohydrates resulting in various biological damages (2).

DNA and RNA undergo oxidation which can cause strand breaks and mutations both of which may be responsible for ageing and carcinogenesis. This oxidation occurs in components of these nucleic acids such as the bases; for instance, guanine base is oxidised in 8-hydroxydeoxyguanisine, a biomarker of DNA damage. Similarly, RNA is more sensitive than DNA as it is composed only of one strand and does not have DNA repair mechanisms. Lipids are also modified by oxidation. This change of lipids causes cell membrane dysfunction and loss of signal transduction due to inactivation of receptor and enzymes (55).

As regards of proteins, many oxidative modifications can occur leading to inactivation, degradation, structure modification and thus disorders in signalling pathways. Modifications are usually based on the sulphur group of cysteine (Cys) and methionine amino acids through thiol oxidation, in the carbonyl group leading to carbonylation and tyrosine amino acids via nitration (3-nitrotyrosine) (2, 16). Indeed, Exposure to excess ROS may result in post-translational modification (PTM), including S-

glutathionylation, S-nitrosylation, acetylation and phosphorylation, which regulate the cellular function (56).

Furthermore, members of the glutathione S-transferase (GST) family's function in the detoxification of various toxic compounds such as carcinogens, drugs, environmental toxins and products of oxidative damage. The GST omega group demonstrates glutaredoxin-like activity, metabolizing reactive oxidants and thereby protecting cells from ROS (57). The GSTs have been extensively studied for their catalytic role in the detoxification of electrophilic compounds and in the removal of endogenously produced free radicals (Reactive Oxygen Species) via their glutathione peroxidase activity (58).

In that regards, we postulated that the CLIC proteins can function as oxidoreductase enzymes within a living cell and could also mediate redox signalling like Trx, Grx and GSTs by performing an ability to oxidize the reduced glutathione (GSH) to disulphides via the reaction of S-glutathionylation. In this study, we used the hydrogen peroxide (H_2O_2), a known oxidative stress inducer. Hydrogen peroxide performs an ability to oxidize the reduced glutathione (GSH) to disulphides through the possibility to exert the reaction of S-glutathionylation (59).

Our findings in Chapter 2 indicate that the CLIC protein family demonstrate glutaredoxin-like enzymatic activity when in their soluble form. In this Chapter, we focused on the oxidoreductase activity within a living cell and we aimed to investigate whether expression of rCLIC proteins by bacterial cells could provide increased tolerance to oxidative stress. The results obtained from these experiments presented for the first time that the CLIC1 afforded the highest level of antioxidant protection within a living cell and can serve to protect cells against oxidative damage and circumvent

oxidative stress, while it was less convincing for CLIC3 and not the case for CLIC4. In thinking about why this might be the case, consider that the various CLICs may likely require specific conditions in order to work optimally – therefore under these current conditions we only really saw an effect by CLIC1 and a lesser effect for CLIC3.

For this purpose, we compare whether expression of rCLIC1 protein by bacterial cells could provide increased tolerance to oxidative stress. As shown in Figure 3.2, within the range of H_2O_2 concentrations tested during this study, the growth of the bacterial cells expressed with the CLIC1-wt protein were higher than the empty cells. Further statistical analysis was carried, and according to ordinary one-way ANOVA multiple comparison the growth rate for the cells transformed with the CLIC1-wt protein, the cells transformed with the empty vector and CLIC1-Cys24S were analysed. The analysis reveals that there is a significant difference in the growth rate of the tested cells within the range of H_2O_2 concentrations tested (the results showed in Figure 3.3).

Moreover, the effect of the H_2O_2 was also analysed on the growth of the bacterial cells expressed with the CLIC1-wt protein and empty cells with respect to time. As expected, the bacterial cells expressed with the CLIC1-wt protein were grown higher than the empty cells at 5.0 mM within 6 hours of investigation Figure 3.6. Additionally, the results in Figure 3.7 reveals the area under curve for each cell line, using Ordinary one-way ANOVA multiple comparison statistical test analysis, CLIC1-wt overexpressing cells present a significantly higher growth rate compared to the cells transformed with pET28a only at 5.0 mM within 6 hours of investigation.

Similarly, where the growth of the bacterial cells expressed with the rCLIC3 protein were grown slightly better than the empty cells within the range of H_2O_2 concentrations tested, Figure 3.8. The statistical analysis showed the absorbance at 600nm for each cell line, using ordinary one-way ANOVA multiple comparison statistical test. It was found

that the CLIC3-wt overexpressing cells did not showed any significant difference in their growth rate when compared to the cells transformed with pGEX-6P-1 vector only, within the range of H_2O_2 concentrations tested Figure 3.9.

In addition to, the growth of the bacterial cells expressed with the rCLIC3-wt protein and the cells transformed with pGEX-6P-1 only were grown higher than the mutant CLIC3-Cys22,25A in pGEX-6P-1 plasmids at 5.0 mM of H2O2 within 6 hours of investigation, Figure 3.10. The results in Figure 3.11 using Ordinary one-way ANOVA statistical test analysis of the absorbance at 600nm calculated for each cell line, indicated that the CLIC3-wt overexpressing cells did not showed any significantly difference in the growth rate when compared to the cells transformed with pGEX-6P-1 only. However, the results showed that there is a higher significant difference in the growth rate of the CLIC3-wt overexpressing cells when it compared to the mutant CLIC3-Cys22,25A in pGEX-6P-1 plasmids at 5.0 mM H2O2 within 6 hours of investigation. *E. coli* cells expressing Cys mutants rCLIC3 Cys22&25 demonstrated much lower tolerance to H2O2 than cells expressing rCLIC3. These results support our hypothesis that the Cys24 in CLIC1 and Cys22&25 in CLIC3 essential for the antioxidant activity and enzymatic function of CLIC proteins.

Furthermore, this could explain the important of the amino acids Cysteines for CLICs proteins structure, folding and enzyme catalysis.

Moreover, the results in Figure 3.12 showed that the growth of the bacterial cells expressed with the rCLIC4-wt protein were to be marginally higher than the empty cells within the range of H_2O_2 concentrations tested. However, the results in Figure 3.13 shows the area under the curve for each cell line, the statistical analysis using ordinary one-way ANOVA multiple comparison statistical test analysis, demonstrates that the

CLIC4-wt overexpressing cells did not show any significant difference in their growth rate cells compared to the cells transformed with pGEX-2T only within the range of H_2O_2 concentrations tested.

In addition, the growth of the bacterial cells expressed with the CLIC4-wt protein and empty cells were appears to grow same or only slightly better than the empty cells at 5.0 mM within 6 hours of investigation Figure3.14. As seen in Figure 3.15, shows the area under the curve for each cell line, we determine the P value using ordinary one-way ANOVA multiple comparison statistical test analysis. Again, the CLIC4-wt overexpressing cells did not showed any significant difference in the growth rate when compared to the cells transformed with the vectors only at 5.0 mM within 6 hours of investigation.

Interestingly, our results showed that of the three rCLICs transformed bacterial cells studied, the cells transformed with rCLIC1 demonstrate highest tolerance to H2O2, compared to either rCLIC3 or rCLIC4. This variation in their oxidoreductase activity within cells could relate to differences determined by cellular localisation and environmental factors. Furthermore, subtle structural differences between these proteins, given the differences in their primary amino acid sequence, would also contribute to differences in activity. We assumed that each CLICs protein may have protective roles under other conditions – but in this study only CLIC1 protein indicates highest level of oxidoreductase activity within cells compared to less convincing CLIC3 proteins and not for the case of CLIC4 proteins. This was postulated to explain differences between CLICs; however, we do not have evidence for this in the bacterial cells.

Taken together, the results in this chapter strongly suggest that CLIC1 and likely CLIC3 function as antioxidants via their oxidoreductase enzymatic activity in cells, capable of protecting the cells against oxidative damage and circumventing oxidative stress.

As previously mentioned in literature review (Chapter1) that the CLIC proteins were highly expressed in different cell types. Multiple physiological functions other than membrane trafficking and cell survival have been ascribed to CLIC family members, and these include their ability to function as scaffolds, their contribution to maintaining cell polarity during cell division as well as a role in cell migration. CLICs have been shown to be enriched at sites comprising large complexes of scaffold and signalling molecules, suggestive of their role as adaptor proteins in the context of many cellular processes.

CLIC2 is widely distributed in human tissues including heart and skeletal muscle (60). It is a strong inhibitor of cardiac ryanodine receptor (RyR) channels and may play an important role in intracellular calcium homeostasis (60). CLIC4 is ubiquitously expressed in different cell and tissue types (61). It is associated with several physiological functions. Of all the CLICs, the MAP kinase associated CLIC3 is the least studied CLIC protein and little is known about its exact function (62).

The p64 was the founding member identified from bovine tracheal epithelium and kidney cortex cells (63, 64). The CLIC5B protein is the human homologue of p64, which is a 46kDa splice variant of CLIC5A (65). CLIC5A was initially identified as a component of a cytoskeleton complex and was later found to function as a chloride ion channel (66). Parchorin is another CLIC protein that was originally identified as a secretory epithelium phosphoprotein in gastric parietal and airway epithelia cells in

rabbits (67). It has been implicated in playing a role in the regulation of secretion by modulating chloride ion channels (60).

Given the plethora of cellular localisations, physiological functions of CLIC proteins and their expression in different cell types indicate that different local conditions would also impact on their differing activity within cells. This high variability of CLICs localisation and distribution could suggest they have different cellular roles within different cell types or multiple roles within a particular cell type.

Previous studies have shown that the transcription factor OxyR in the *E.coli* Grx1 functions as a redox sensor by forming an intramolecular disulphide bond when the environment becomes oxidised. The oxidised OxyR activates the expression of antioxidant genes involved in the response to the H_2O_2 and is subsequently inactivated by enzymatic reduction of the disulphide by *E.coli* Grx1 (68). Thus the evolutionary conservation in *E.coli* of a disulphide activated transcription system as a key response system to hydrogen peroxide makes the presence of such system highly plausible. Furthermore, GSH also plays an indirect role in cells under peroxide stress by reducing oxidized OxyR in a similar manner to glutaredoxin 1. Thus, when the oxidative challenge has passed, OxyR is restored to its reduced and transcriptionally inactive state (69).

In bacterial cells, the physiological concentration of GSH ranges from 0.1 to about 10 mM. In *Escherichia coli* the glutathione content increased significantly during transition from exponential to stationary phase. Because of its two carboxyls, one

amine, and one thiol group, GSH is highly soluble in aqueous solutions and in polar solvents.

Furthermore in *E. coli*, the GSH plays a critical role in protection against environmental stresses which include osmotic shock, acidity, protection against toxins like methylglyoxal, chlorine compounds like hypochlorous acid and monochloroamine, and oxidative stress induced by peroxides, such as hydrogen peroxide (H_2O_2) or alkyl hydroperoxides. Also, the GSH is involved in the regulation of intracellular potassium levels and in preventing the formation of aberrant protein disulfides in the cytoplasm (69).

Moreover, some of the prokaryotes that lack glutathione, but they seem to produce another different low molecular weight thiol which appear to function in a similar way to GSH. For example, anaerobic sulfur bacteria use glutathione amide, while the major thiols in aerobic phototrophic halobacteria and in actinomycetes are ɣ-glutamylcysteine and mycothiol, respectively (70, 71).

Indeed, the most important mechanisms of transducing oxidant dependent signals are through the amino acid cysteine (Cys, C) due to its sulfhydryl side chain (SH). However, only certain cysteine residues have been denoted as ‘reactive’, meaning they can be readily oxidized, reduced and otherwise modified. This microenvironment favours the loss of a proton from the SH group of cysteine, forming a thiolate anion (S-) which can then be oxidized to sulfenic acid (SOH), the initial oxidation product of cysteine (72).

The addition of the external H_2O_2 would generate a concentration gradient through cell membrane (73), building up the oxidative environment to mimic oxidative stress. Inside cells, the environment is normally reduced due to high levels of GSH (59). However,

oxidative stress can alter this environment allowing exposed cellular thiols to become oxidized (74). Thus, thiol oxidation causing a significant production of reactive oxygen species (ROS) (75). In turn, increased generation of ROS and superoxides leads to cellular damage and can induce apoptosis. The introduction of the oxidant (Hydrogen Peroxide) would result in oxidation of thiol groups, specifically on cysteine residues (16, 59), along with other susceptible reactive groups within cells. Furthermore, in the process of stress disposal, oxidative stress leads to the accumulation of GSSG from the reaction between GSH and H_2O_2. The excessive production of GSSG facilitates the S-glutathionylation of proteins, making them as cellular targets of oxidative stress (76, 77).

Therefore, we speculate that the CLIC1 proteins function as antioxidant and oxidoreductase enzymes within the cells and less convincing for the CLIC3 proteins and not on the case of CLIC4 proteins, its ability to do this via its active site motif (Cysteine residues), (the monothiol Cys24 in CLIC1 and dithiol Cys 22,25 in CLIC3).

Thus, we are looking at its ability to reduce other substrates and investigate the effect of the oxidant molecule H_2O_2 on the growth rate of the bacterial cells. In this study, we mutated the Cys24 in the CLIC1 to serine and the Cys22,25 in the CLIC3 to alanine, by challenging these mutants to the oxidant (H_2O_2) at different concentrations of the oxidant (2.5, 5.0 and 10.0 mM) after 3 hours of incubation. Also, at the same concentration (5 mM) over increasing a time of period within 6 hours of investigation. We found that the cysteine 24 residue in the CLIC1 structure was susceptible to oxidation as seen in Figure 3.2 and Figure 3.6, that the growth rate of the E. *coli* BL21 (DE3) rCLIC1-Cys24S mutant cells significantly lower than the E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC1-wt protein.

In contrast, both cysteine 22,25 residues in the CLIC3 structure was susceptible to oxidation. Results showed in Figure 3.8 and Figure 3.10, that the growth rate of the mutant CLIC3-Cys22,25A cells decreased when it compared to the E. *coli* BL21 (DE3) bacterial cells transformed to express the CLIC3-wt protein.

This result assign that the CLIC1 protein which contain monothiol G-active site, act as a monothiol, while the CLIC3 contain a dithiol G-active site and act as a dithiol of oxidative stress and redox regulation. This active site coupled with the Glutathione peptide (GSH) that is used as a cofactor in the redox reactions catalysed by members of the Grx family. According to this activity, the disulphide bond in protein targets and other component substrates are reversibly reduced, leading to maintain a healthy reduced state within cells intracellular environment (16). However, further studies are needed in order to establish a distinct dithiol catalytic mechanism by CLIC2 and CLIC3 compared to the monothiol members CLIC1 and CLIC4 (78).

So far, to conclude the results presented in this study demonstrate for the first time that rCLIC1 showed the highest level of antioxidant and oxidoreductase enzymes within prokaryotic cells when it compared to rCLIC3 and rCLIC4. However, further investigations would be necessary to explain the role played by these proteins in the regulations and the relevance in general cell metabolism. It is important to keep in mind that the extent of modified protein can be a minor part of total cellular pool, and that these modifications might not be relevant for the regulation of the pathway. Indeed, several proteins, as moonlighting protein that are diverted, after modifications, to different functions. This enzymatic versatility is regulated in part by cysteine-based redox modifications that could alter the CLIC activity and its sub-cellular localization. Generally, these modifications occur under stress conditions, but they might be relevant

also under normal conditions. Considering the central role of CLICs proteins in different cellular metabolism also their expression in versatile cellular localization could help understanding of this complex regulation is a challenge for the future.

Furthermore, our results strongly suggest that the CLICs proteins contains cysteines sensitive to redox modifications, in particular Cys-24S in CLIC1 and Cys-22,25A in CLIC3, these Cysteines are target of S-glutathionylation. It will be interesting to understand the interplay between the modifications and analyse how these modifications affect the protein activity *in vivo* under stress conditions. Considering that the structural features for the redox regulation are directly involved in determination of the type and extent of redox modifications, by solving the 3D-structure of the protein could be a powerful tool to analyse the protein microenvironment of each cysteine residue that influence the sensitivity to different type of redox modifications.

To summarise, our results strength the proposal that the soluble CLIC1 proteins could act as general antioxidant and an oxidoreductase enzyme in cells. This suggest an additional role of CLICs proteins in the cellular processes of detoxification and oxidoreduction. This is also supported by the structural evidences that the CLICs proteins have a high level of structural and functional similarity with the GST-Ω, Grxs and Trxs with the latter three groups have well-known oxidoreductase class of enzymes.

3. 9 Concluding Remarks

The imbalance between the production of ROS and protective reducing environment can cause toxic effects through the overproduced free radicals and peroxides, which may damage the cellular components, such as lipids, DNA and proteins. When the production of the reactive oxygen species (ROS) exceeds the cleaning ability of the cellular antioxidant system, the oxidative stress occurs. S-glutathionylation by the addition of small chemical groups. Within these posttranslational modifications, S-glutathionylation is what we are most concerned.

Previous data from our group indicated that the CLICs demonstrate glutaredoxin-like enzymatic activity when in their soluble form (78). In the current study, we found that the protein CLIC1 and to a lesser extent CLIC3, act as cell protective proteins and possesses a cellular antioxidant activity. We observed significantly higher tolerance to different concentrations of the oxidant H_2O_2 in bacterial *E. coli* cells expressing CLIC1 in comparison to the respective control bacterial *E. coli* cells. Interestingly, the mutant forms of the proteins lacking the critical cysteine residue in the enzymatic active site did not. Although further experiments are required to elucidate the mechanism through CLICs proteins cellular antioxidant activity. However, this study demonstrates for the first time the *direct* cellular antioxidant activity by CLIC1 and confirms it is a moonlighting protein, with two distinct functions – a membrane ion channel and an antioxidant oxidoreductase enzyme.

The findings of this chapter added a small piece to the big complex picture of redox regulation and may inspire others for future studies, because it is evident that ROS play essential roles in both physiological and pathological conditions. Also, the ROS system

is important for defending, cellular detoxification and redox signalling. To summarize, this chapter investigated the role of CLIC proteins to protect the cells under oxidative stress.

3. 10 References

1. **Madamanchi NR, Vendrov A, Runge MS.** 2005. Oxidative stress and vascular disease. Arteriosclerosis, thrombosis, and vascular biology **25:**29-38.
2. **Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J.** 2007. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology **39:**44-84.
3. **Ghezzi P.** 2005. Oxidoreduction of protein thiols in redox regulation. Biochemical Society Transactions **33:**1378-1381.
4. **Ferrer-Sueta G, Manta B, Botti H, Radi R, Trujillo M, Denicola A.** 2011. Factors affecting protein thiol reactivity and specificity in peroxide reduction. Chemical research in toxicology **24:**434-450.
5. **Ying J, Clavreul N, Sethuraman M, Adachi T, Cohen RA.** 2007. Thiol oxidation in signaling and response to stress: detection and quantification of physiological and pathophysiological thiol modifications. Free Radical Biology and Medicine **43:**1099-1108.
6. **Stubbs CD, Smith AD.** 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes **779:**89-137.

7. **Halliwell B, Chirico S.** 1993. Lipid peroxidation: its mechanism, measurement, and significance. The American journal of clinical nutrition **57:**715S-725S.

8. **Pogozelski WK, Tullius TD.** 1998. Oxidative strand scission of nucleic acids: routes initiated by hydrogen abstraction from the sugar moiety. Chemical reviews **98:**1089-1108.

9. **O'Driscoll M, Jeggo PA.** 2006. The role of double-strand break repair—insights from human genetics. Nature Reviews Genetics **7:**45.

10. **Roos WP, Kaina B.** 2006. DNA damage-induced cell death by apoptosis. Trends in molecular medicine **12:**440-450.

11. **Sato Y, Tsurumi T.** 2013. Genome guardian p53 and viral infections. Reviews in medical virology **23:**213-220.

12. **Cao X, Kambe F, Lu X, Kobayashi N, Ohmori S, Seo H.** 2005. Glutathionylation of two cysteine residues in paired domain regulates DNA binding activity of Pax-8. Journal of Biological Chemistry **280:**25901-25906.

13. **Halliwell B.** 2007. Biochemistry of oxidative stress. Portland Press Limited.

14. **Halliwell B.** 2013. The antioxidant paradox: less paradoxical now? British Journal of Clinical Pharmacology **75:**637-644.

15. **Laurent TC, Moore EC, Reichard P.** 1964. Enzymatic synthesis of deoxyribonucleotides IV. Isolation and characterization of thioredoxin, the hydrogen donor from Escherichia coli B. Journal of Biological Chemistry **239:**3436-3444.

16. **Hanschmann E-M, Godoy JR, Berndt C, Hudemann C, Lillig CH.** 2013. Thioredoxins, glutaredoxins, and peroxiredoxins—molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. Antioxidants & redox signaling **19:**1539-1605.

17. **Holmgren A, Söderberg B, Eklund H, Brändén C.** 1975. Three-dimensional structure of Escherichia coli thioredoxin-S2 to 2.8 A resolution. Proceedings of the National Academy of Sciences **72:**2305-2309.

18. **Collet J-F, Messens J.** 2010. Structure, function, and mechanism of thioredoxin proteins. Antioxidants & redox signaling **13:**1205-1216.

19. **Vlamis-Gardikas A, Holmgren A.** 2002. Thioredoxin and glutaredoxin isoforms. Methods in enzymology **347:**286-296.

20. **Miranda-Vizuete A, Sadek CM, Jiménez A, Krause WJ, Sutovsky P, Oko R.** 2004. The mammalian testis-specific thioredoxin system. Antioxidants and Redox Signaling **6:**25-40.

21. **Lillig CH, Holmgren A.** 2007. Thioredoxin and related molecules–from biology to health and disease. Antioxidants & redox signaling **9:**25-47.

22. **Haendeler J.** 2006. Thioredoxin-1 and posttranslational modifications. Antioxidants & redox signaling **8:**1723-1728.

23. **Fernandes AP, Holmgren A.** 2004. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxidants and Redox Signaling **6:**63-74.

24. **Holmgren A.** 1976. Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. Proceedings of the National Academy of Sciences **73:**2275-2279.

25. **Holmgren A.** 1979. Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli. Journal of Biological Chemistry **254:**3664-3671.

26. **Höög JO, Jörnvall H, Holmgren A, Carlquist M, Persson M.** 1983. The primary structure of Escherichia coli glutaredoxin: Distant homology with

thioredoxins in a superfamily of small proteins with a redox-active cystine disulfide/cysteine dithiol. European journal of biochemistry **136:**223-232.

27. **Luthman M, Eriksson S, Holmgren A, Thelander L.** 1979. Glutathione-dependent hydrogen donor system for calf thymus ribonucleoside-diphosphate reductase. Proceedings of the National Academy of Sciences **76:**2158-2162.

28. **Luthman M, Holmgren A.** 1982. Glutaredoxin from calf thymus. Purification to homogeneity. Journal of Biological Chemistry **257:**6686-6690.

29. **Padilla CA, Martínez-Galisteo E, López-Barea J, Holmgren A, Bárcena JA.** 1992. Immunolocalization of thioredoxin and glutaredoxin in mammalian hypophysis. Molecular and cellular endocrinology **85:**1-12.

30. **Rozell B, Bárcena JA, Martinez-Galisteo E, Padilla CA, Holmgren A.** 1993. Immunochemical characterization and tissue distribution of glutaredoxin (thioltransferase) from calf. European journal of cell biology **62:**314-323.

31. **Pai HV, Starke DW, Lesnefsky EJ, Hoppel CL, Mieyal JJ.** 2007. What is the functional significance of the unique location of glutaredoxin 1 (GRx1) in the intermembrane space of mitochondria? Antioxidants & redox signaling **9:**2027-2034.

32. **Lundberg M, Fernandes AP, Kumar S, Holmgren A.** 2004. Cellular and plasma levels of human glutaredoxin 1 and 2 detected by sensitive ELISA systems. Biochemical and biophysical research communications **319:**801-809.

33. **Aesif SW, Anathy V, Kuipers I, Guala AS, Reiss JN, Ho Y-S, Janssen-Heininger YM.** 2011. Ablation of glutaredoxin-1 attenuates lipopolysaccharide-induced lung inflammation and alveolar macrophage activation. American journal of respiratory cell and molecular biology **44:**491-499.

34. **Bachschmid MM, Xu S, Maitland-Toolan KA, Ho Y-S, Cohen RA, Matsui R.** 2010. Attenuated cardiovascular hypertrophy and oxidant generation in response to angiotensin II infusion in glutaredoxin-1 knockout mice. Free Radical Biology and Medicine **49:**1221-1229.

35. **Hoffman S, Godburn K, Guala A, Sheely J, Ho Y-S, Irvin CG, Lundblad LK, Aliyeva M, Daphtary N, Janssen-Heininger YM.** 2011. Genetic Ablation Of Glutaredoxin-1 Causes An Enhanced Resolution Of Airways Hyper Responsiveness (AHR) In Mice In Association With Increases In S-Glutathionylation, p A2572-A2572, B22 MECHANISMS OF AIRWAY HYPERRESPONSIVENESS IN ASTHMA. American Thoracic Society.

36. **Johansson C, Lillig CH, Holmgren A.** 2004. Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. Journal of Biological Chemistry **279:**7537-7543.

37. **Lillig CH, Berndt C, Vergnolle O, Lönn ME, Hudemann C, Bill E, Holmgren A.** 2005. Characterization of human glutaredoxin 2 as iron–sulfur protein: a possible role as redox sensor. Proceedings of the National Academy of Sciences **102:**8168-8173.

38. **Wu H, Lin L, Giblin F, Ho Y-S, Lou MF.** 2011. Glutaredoxin 2 knockout increases sensitivity to oxidative stress in mouse lens epithelial cells. Free Radical Biology and Medicine **51:**2108-2117.

39. **Mailloux RJ, Xuan JY, McBride S, Maharsy W, Thorn S, Holterman CE, Kennedy CR, Rippstein P, Da Silva J, Nemer M.** 2014. Glutaredoxin-2 is required to control oxidative phosphorylation in cardiac muscle by mediating

deglutathionylation reactions. Journal of Biological Chemistry:jbc. M114. 550574.

40. **Isakov N, Witte S, Altman A.** 2000. PICOT-HD: a highly conserved protein domain that is often associated with thioredoxin and glutaredoxin modules. Trends in biochemical sciences **25:**537-539.

41. **Cha H, Kim JM, Oh JG, Jeong MH, Park CS, Park J, Jeong HJ, Park BK, Lee Y-H, Jeong D.** 2008. PICOT is a critical regulator of cardiac hypertrophy and cardiomyocyte contractility. Journal of molecular and cellular cardiology **45:**796-803.

42. **Haunhorst P, Berndt C, Eitner S, Godoy JR, Lillig CH.** 2010. Characterization of the human monothiol glutaredoxin 3 (PICOT) as iron–sulfur protein. Biochemical and biophysical research communications **394:**372-376.

43. **Wingert RA, Galloway JL, Barut B, Foott H, Fraenkel P, Axe JL, Weber GJ, Dooley K, Davidson AJ, Schmidt B.** 2005. Deficiency of glutaredoxin 5 reveals Fe–S clusters are required for vertebrate haem synthesis. Nature **436:**1035.

44. **Ye H, Jeong SY, Ghosh MC, Kovtunovych G, Silvestri L, Ortillo D, Uchida N, Tisdale J, Camaschella C, Rouault TA.** 2010. Glutaredoxin 5 deficiency causes sideroblastic anemia by specifically impairing heme biosynthesis and depleting cytosolic iron in human erythroblasts. The Journal of clinical investigation **120:**1749-1761.

45. **Camaschella C, Campanella A, De Falco L, Boschetto L, Merlini R, Silvestri L, Levi S, Iolascon A.** 2007. The human counterpart of zebrafish shiraz shows sideroblastic-like microcytic anemia and iron overload. Blood **110:**1353-1358.

46. **Peltoniemi MJ, Karala A-R, Jurvansuu JK, Kinnula VL, Ruddock LW.** 2006. Insights into Deglutathionylation Reactions DIFFERENT INTERMEDIATES IN THE GLUTAREDOXIN AND PROTEIN DISULFIDE ISOMERASE CATALYZED REACTIONS ARE DEFINED BY THE γ-LINKAGE PRESENT IN GLUTATHIONE. Journal of Biological Chemistry **281:**33107-33114.

47. **Findlay VJ, Townsend DM, Morris TE, Fraser JP, He L, Tew KD.** 2006. A novel role for human sulfiredoxin in the reversal of glutathionylation. Cancer research **66:**6800-6806.

48. **Ghezzi P, Bonetto V.** 2003. Redox proteomics: identification of oxidatively modified proteins. PROTEOMICS: International Edition **3:**1145-1153.

49. **Weissbach H, Resnick L, Brot N.** 2005. Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics **1703:**203-212.

50. **Dalle-Donne I, Giustarini D, Rossi R, Colombo R, Milzani A.** 2003. Reversible S-glutathionylation of Cys374 regulates actin filament formation by inducing structural changes in the actin molecule. Free Radical Biology and Medicine **34:**23-32.

51. **Dalle-Donne I, Rossi R, Colombo G, Giustarini D, Milzani A.** 2009. Protein S-glutathionylation: a regulatory device from bacteria to humans. Trends in biochemical sciences **34:**85-96.

52. **Hasenbrink G, Schwarzer S, Kolacna L, Ludwig J, Sychrova H, Lichtenberg-Fraté H.** 2005. Analysis of the mKir2. 1 channel activity in potassium influx defective Saccharomyces cerevisiae strains determined as changes in growth characteristics. FEBS letters **579:**1723-1731.

53. **Anathy V, Roberson EC, Guala AS, Godburn KE, Budd RC, Janssen-Heininger YM.** 2012. Redox-based regulation of apoptosis: S-glutathionylation as a regulatory mechanism to control cell death. Antioxidants & redox signaling **16:**496-505.

54. **Hill BG, Bhatnagar A.** 2012. Protein S-glutathiolation: redox-sensitive regulation of protein function. Journal of molecular and cellular cardiology **52:**559-567.

55. **Esterbauer H, Schaur RJ, Zollner H.** 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free radical Biology and medicine **11:**81-128.

56. **Menon D, Board PG.** 2013. A role for glutathione transferase Omega 1 (GSTO1-1) in the glutathionylation cycle. Journal of Biological Chemistry **288:**25769-25779.

57. **Listowsky I.** 2005. A Subclass of Mu Glutathione S-Transferases Selectively Expressed in Testis and Brain. Methods in enzymology **401:**278-287.

58. **Hayes JD, Flanagan JU, Jowsey IR.** 2005. Glutathione transferases. Annu Rev Pharmacol Toxicol **45:**51-88.

59. **Li M, Huang W, Yang Q, Liu X, Wu Q.** 2005. Expression and oxidative stress tolerance studies of glutaredoxin from cyanobacterium Synechocystis sp. PCC 6803 in Escherichia coli. Protein expression and purification **42:**85-91.

60. **Board PG, Coggan M, Watson S, Gage PW, Dulhunty AF.** 2004. CLIC-2 modulates cardiac ryanodine receptor Ca 2+ release channels. The international journal of biochemistry & cell biology **36:**1599-1612.

61. **Howell S, Duncan RR, Ashley RH.** 1996. Identification and characterisation of a homologue of p64 in rat tissues. FEBS letters **390:**207-210.

62. **Qian Z, Okuhara D, Abe MK, Rosner MR.** 1999. Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. Journal of Biological Chemistry **274:**1621-1627.

63. **Landry D, Sullivan S, Nicolaides M, Redhead C, Edelman A, Field M, al-Awqati Q, Edwards J.** 1993. Molecular cloning and characterization of p64, a chloride channel protein from kidney microsomes. Journal of Biological Chemistry **268:**14948-14955.

64. **Redhead CR, Edelman AE, Brown D, Landry DW, Al-Awqati Q.** 1992. A ubiquitous 64-kDa protein is a component of a chloride channel of plasma and intracellular membranes. Proceedings of the National Academy of Sciences **89:**3716-3720.

65. **Shanks RA, Larocca MC, Berryman M, Edwards JC, Urushidani T, Navarre J, Goldenring JR.** 2002. AKAP350 at the Golgi Apparatus II. ASSOCIATION OF AKAP350 WITH A NOVEL CHLORIDE INTRACELLULAR CHANNEL (CLIC) FAMILY MEMBER. Journal of Biological Chemistry **277:**40973-40980.

66. **Berryman M, Bretscher A.** 2000. Identification of a novel member of the chloride intracellular channel gene family (CLIC5) that associates with the actin cytoskeleton of placental microvilli. Molecular biology of the cell **11:**1509-1521.

67. **Nishizawa T, Nagao T, Iwatsubo T, Forte JG, Urushidani T.** 2000. Molecular cloning and characterization of a novel chloride intracellular channel-related protein, parchorin, expressed in water-secreting cells. Journal of Biological Chemistry **275:**11164-11173.

68. **Zheng M, Åslund F, Storz G.** 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science **279:**1718-1722.

69. **Masip L, Veeravalli K, Georgiou G.** 2006. The many faces of glutathione in bacteria. Antioxidants & redox signaling **8:**753-762.

70. **Newton GL, Arnold K, Price MS, Sherrill C, Delcardayre SB, Aharonowitz Y, Cohen G, Davies J, Fahey RC, Davis C.** 1996. Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. Journal of bacteriology **178:**1990-1995.

71. **Fahey RC.** 2001. Novel thiols of prokaryotes. Annual Reviews in Microbiology **55:**333-356.

72. **Finkel T.** 2011. Signal transduction by reactive oxygen species. The Journal of cell biology **194:**7-15.

73. **Bienert GP, Schjoerring JK, Jahn TP.** 2006. Membrane transport of hydrogen peroxide. Biochimica et Biophysica Acta (BBA)-Biomembranes **1758:**994-1003.

74. **Chrestensen CA, Eckman CB, Starke DW, Mieyal JJ.** 1995. Cloning, expression and characterization of human thioltransferase (glutaredoxin) in E. coli. FEBS letters **374:**25-28.

75. **Björnstedt M, Kumar S, Holmgren A.** 1994. Selenite and selenodiglutathione: reactions with thioredoxin systems. Methods in enzymology **252:**209-219.

76. **Mieyal JJ, Gallogly, M. M., Qanungo, S., Sabens, E. A., & Shelton, M. D. .** 2008. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. . Antioxidants and Redox Signaling, **10** 1941-1988.

77. **Sabens Liedhegner EA, Gao X-H, Mieyal JJ.** 2012. Mechanisms of altered redox regulation in neurodegenerative diseases—focus on S-glutathionylation. Antioxidants & redox signaling **16:**543-566.

78. **Al Khamici H, Brown LJ, Hossain KR, Hudson AL, Sinclair-Burton AA, Ng JPM, Daniel EL, Hare JE, Cornell BA, Curmi PM.** 2015. Members of the Chloride Intracellular Ion Channel Protein Family Demonstrate Glutaredoxin-Like Enzymatic Activity. PloS one **10:**e115699.

Chapter 4

Members of the Chloride Intracellular Ion Channel Protein Family Catalyse Protein Deglutathionylation

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Chapter 4

4. 1 Introduction

The process of protein glutathionylation provides a primary line of defense against irreversible protein damage caused by oxidative stress. Glutathionylation is defined as a post translational modification (PTM) of protein thiol groups via the formation of mixed disulphides with glutathione (1). On oxidative insult, redox sensitive protein thiol groups are glutathionylated (capping cysteine residues), thus shielding them from irreversible oxidative damage. This reaction may occur spontaneously, but growing evidence suggests the catalytic involvement of the Glutaredoxin (Grx) family thioltransferases (2). Additionally, glutathionylation reactions may also be reversed via deglutathionylation, specifically, removal of protein bound glutathione. Glutaredoxins and sulfiredoxin are known to catalyse the deglutathionylation of proteins and thus protect protein thiols from oxidation to either the oxidized sulfenic acid or sulfinic acid forms (3). Thus, protein thiol groups can be transiently capped with glutathione until the redox balance is restored, at which time proteins can be enzymatically deglutathionylated. This makes glutathione an indispensable player in the regulation of cellular redox homeostasis. Aside from its well characterised role in oxidative stress, reversible glutathionylation in the absence of oxidative stress, as a post-translational protein modification, has also been shown to affect protein function. Indeed, glutathionylation has been shown to have an impact on the regulation of the cell cycle, apoptosis and inflammation (4). Therefore, the mechanisms and enzymatic mediators involved in the glutathionylation cycle are of great interest under both oxidative and non-oxidative conditions.

Most GSTs possess a tyrosine or serine residue at their active site, responsible for catalysing conjugation reactions between GSH and electrophilic substrates. Previous data

have confirmed that GSTO1-1 contributes to protein glutathionylation and deglutathionylation reactions (1). Due to the CLIC protein members having structural similarity to the GSTO1-1, we postulate that CLIC proteins may also contribute to glutathionylation and deglutathionylation reactions. The CLIC's crystal structure is also suggestive of a novel role for the CLIC proteins in the glutathionylation cycle, as their active site is "open", and thus could accommodate large substrates, such as proteins (5, 6). Based on these recent findings, my project proposes that CLICs proteins may regulate a variety of target proteins via a common redox sensitive mechanism. In this chapter, the ability of CLIC proteins to undertake glutathionylation and deglutathionylation reactions has been investigated.

4. 2 Redox Regulation

The change of redox-state of thiols can occur after challenge by environmental stresses, in order to maintain cellular homeostasis and prevent oxidative damage (7). The oxidative burst by these stresses depends on accumulation of reactive oxygen species (ROS) including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$), together with singlet oxygen ($^1O_2$) (8). Among amino acids, the most oxidation-susceptible residues are the sulphur containing ones, cysteine and methionine (9). Although the cysteine residue is one of the least abundant amino acids, it plays important roles in reversible regulation, through change in the redox state of proteins, followed by changes in structural, catalytic and regulatory functions (8, 10). Cysteinyl thiols are particularly susceptible to oxidative modifications and can undergo a broad spectrum of redox reactions that are dependent on the species and concentration of oxidants they contact (11). Indeed, the formation of disulfide bridges through the Trx-system may

contribute to maintenance of protein structural stability, regulation of enzymatic activity and binding of cofactors or other proteins (1). Trx regulation is not the only regulation of thiol groups, with the cysteines also able to undergo reversible glutathionylation (12, 13).

Several antioxidant defense systems are important in combating ROS production and include enzymatic and non-enzymatic mechanisms. The major intracellular antioxidant is the non-protein thiol glutathione (GSH). Glutathione exists in virtually all cells in the millimolar concentration range and it is the most abundant non-protein thiol present in cells (14-16). Glutathione is involved in various cellular process including growth, cell differentiation, cell cycle progression, transcriptional activity, pathogen resistance, cytoskeletal functions, and metabolism, stomatal closure, signaling protein, energy metabolism, protein folding and degradation (14-17). Moreover, glutathione is involved in the cysteine-based redox modification S-glutathionylation, consisting in the formation of a transient mixed disulfide between an accessible protein thiol and one molecule of glutathione (1). Glutathione is a tripeptide antioxidant consisting of -glutamyl-cysteinyl-glycine synthesized by -glutamylcysteinyl synthetase (18, 19) (Figure 4.1). The glutathione redox cycle involves cycling between reduced glutathione (GSH) and oxidized glutathione (GSSG), with the reduction of GSSG occurring via the action of the enzyme glutathione reductase (2, 20). The GSH/GSSG ratio is used as a good indicator of intracellular redox status (21). Several other enzymes use GSH to detoxify radical species, including glutathione peroxidase and glutathione S-transferases (22). Because of these defenses, H_2O_2 can be tolerated by cells to micromolar concentrations before it becomes lethal (22). Moreover, excess production of ROS or inhibition of antioxidant defense systems can result in an imbalance, leading to oxidative stress (7, 22).

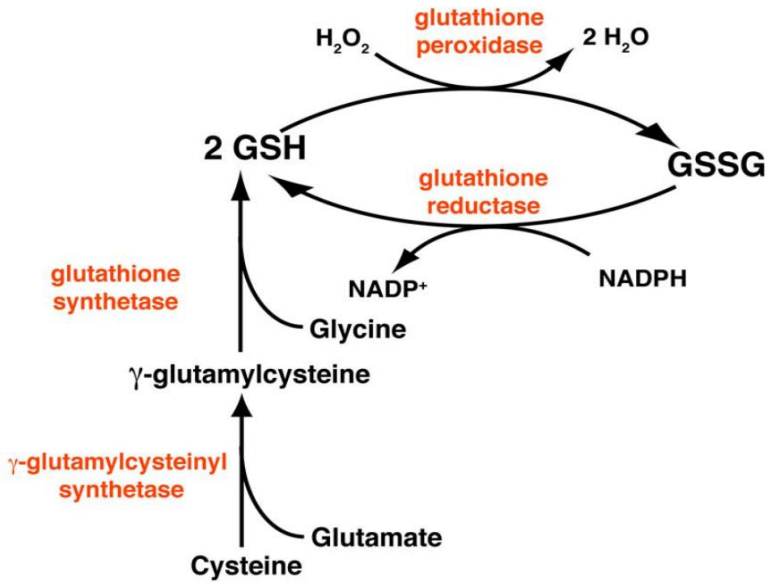

Figure 4. 1 Glutathione redox cycle. The first step in the biosynthesis of GSH involves the conjugation of the -carbon of glutamate to cysteine, via the enzyme -glutamylcysteinyl synthetase, which is the rate-limiting step. *The final step involves addition of glycine. The resulting reduced GSH can participate in the glutathione redox cycle. Oxidation of GSH by ROS converts 2 GSH molecules into GSSG. This process can be catalysed by enzymes, such as glutathione peroxidase. Glutathione reductase can use the reductant NADPH to reduce GSSG back to GSH, completing the cycle (18). Enzymes are in red. Figure adapted from (18).*

4. 3 Protein S-glutathionylation

4.3. 5 Mechanisms of glutathionylation

Redox status is now recognized as a means of regulating various proteins and enzymes under physiological and pathological conditions (4). One prominent modification is reversible protein S-glutathionylation, a post-translational modification of proteins involving formation of a mixed disulfide between a protein cysteinyl residue and GSH.

This occurs in response to oxidative stress and may be a mechanism to protect proteins from irreversible oxidative damage (23). Oxidation of protein thiols can result in the formation of sulfenic acids (-SOH), which can be further oxidized to sulfinic (-SO2H) or sulfonic acids (-SO3H) (4). Protein glutathionylation can also occur in the absence of exogenous oxidative stress, and may be a mechanism for redox regulation of protein function under physiological conditions (4, 24). Interestingly, increases in protein S-glutathionylation have been shown to occur in various disease states, including hyperlipidemia, diabetes mellitus and chronic renal failure (4). The exact mechanisms of protein S-glutathionylation are still the subject of much debate. They include thiol-disulfide exchange, sulfenic acid intermediates, thiyl radical intermediates, and S-nitrosylated intermediates (Figure 4.2A). Thiol-disulfide exchange depends on the GSH/GSSG ratio and may occur through an exchange between GSSG and protein thiol exchange. More plausible mechanisms involve reactive thiol derivatives including sulfenic acids or thiyl radical intermediates. Sulfenic acids formed under physiological conditions, are highly unstable and rapidly undergo further oxidation to sulfinic or sulfonic acids. Sulfenic acids are believed to be the major protein intermediates that are readily glutathionylated. Thiyl radicals are amongst the shortest-lived sulfhydryl derivatives and form glutathionylated proteins through radical recombination or reaction with a thiolate and O_2 (23, 25). The major enzyme that has been shown to catalyse the reactions between GSH intermediates and protein S-glutathionylation is glutaredoxin (Grx). The Glutaredoxin proteins are part of the family of thioltransferases and have been shown to be the major deglutathionylating enzymes (4). Due to the low pKa of the active site-cysteine, Grx may catalyse protein glutathionyation through stabilization of the glutathione thiyl radical, allowing the active site-cysteine to become glutathionylated. It

is then reduced back to its original state by GSH (Figure 4.2B). Interestingly, under some circumstances, inhibition of Grx resulted in increased protein glutathionylation (26). Therefore, it is likely that the redox state of the cell contributes to the de / glutathionylating activity of Grx. It also suggests involvement of other proteins in cellular glutathionylation.

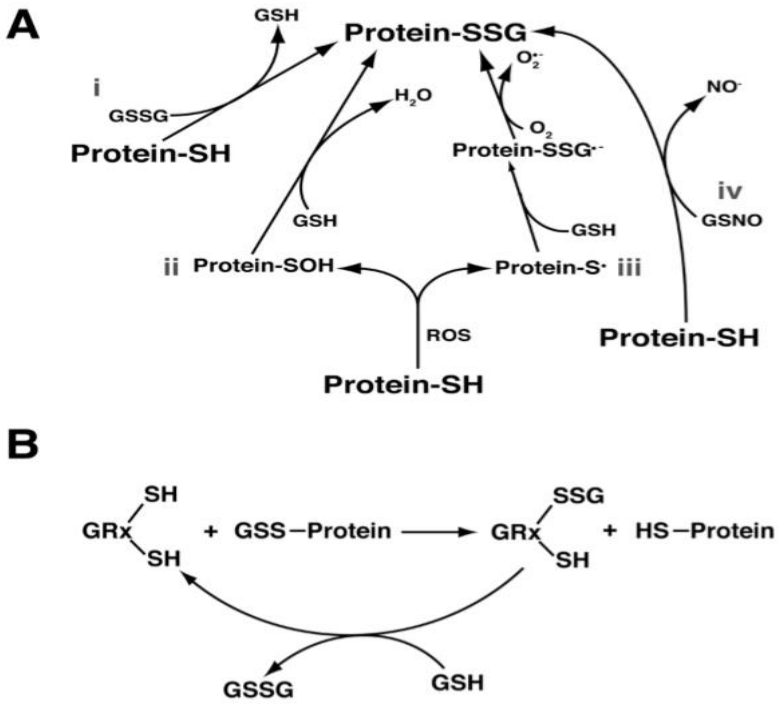

Figure 4. 2 Mechanisms of protein S-glutathionylation. *A) i) Thiol-disulfide exchange is dependent on GSH/GSSG ratio and oxidation potential for formation of mixed disulfide (protein-SSG). ii) Sulfenic acid intermediates (-SOH) and iii) thiyl radical intermediates (RS•) that form as a result endogenously produced ROS are highly unstable and susceptible to reduction by GSH. iv) S-nitrosylated intermediates can form GSNO (shown) or proteinSNO formation. These intermediates are more stable than the oxygen intermediates in ii) and iii) but have been shown to readily react with a variety of proteins resulting protein-SSG formation. B) Glutaredoxin mechanism of action involves a first*

step monothiol-disulfide exchange between the GRx active site and the glutathionylated sulfur moiety of protein-SSG resulting in formation of protein-SH. The second step involves reduction of GRx-SSG by GSH to produce GSSG as the second product, recycling the reduced enzyme (4). Figure adapted from Mieyl et al. (4).

4.3. 6 Deglutathionylation of target proteins

The de/glutathionylation cycle is a significant mechanism in redox regulation and signaling processes (27). Glutathionylation of protein targets can be reversed by deglutathionylation (28), where the GSH is removed from the thiol groups of protein cysteine residues, consequently regenerating the activities performed by these proteins (Figure 4.3) (29).

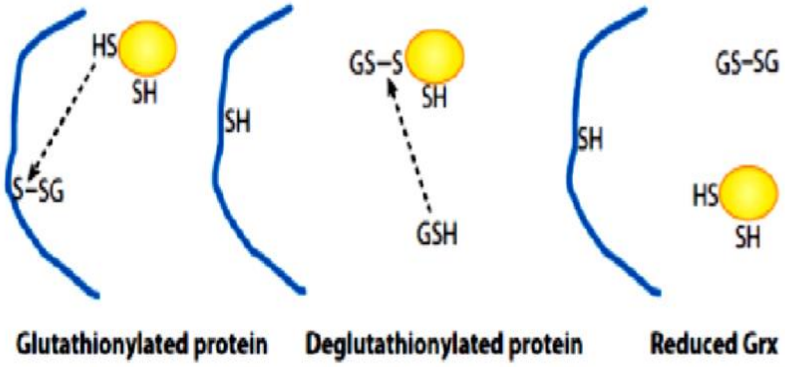

Figure 4. 3 Deglutathionylation of a target protein by glutaredoxin (Grx). *Reduced glutaredoxin (yellow circle) removes glutathione (GSH) from the glutathionylated protein and is subsequently reduced by GSH (13, 29).*

Though deglutathionylation was originally identified as a mechanism to reverse oxidative stress induced glutathionylation, there is increasing evidence indicating that some proteins can be actively deglutathionylated in response to oxidative stress (2). The synergistic activity of glutathione, glutaredoxins and thioredoxin may be considered as the major gatekeepers of the cellular cysteine thiol pool (30). In addition, numerous dithiol glutaredoxins are capable of catalysing the GSH dependent reduction of disulfides or glutathionylated cysteines and are in turn reduced by GSH (29). Non-enzymatic glutathionylation occurs under oxidative stress without substrate specificity. On the contrary, deglutathionylation is tightly regulated by enzymes with target specificity even under normal physiological redox conditions (26, 28).

Deglutathionylation is predominantly catalysed by the glutaredoxin family of thioltransferases though other enzymes sharing the typical 'thioredoxin' fold such as GSTO and more recently the CLIC proteins, are gradually being characterized with similar glutaredoxin like activity (1, 31).

4.3. 7 Protein specificity of enzymes catalysing the glutathionylation cycle

The specificity of protein S-glutathionylation has not been fully understood and is still under wide debate (32). As such, the parameters defining the specificity of enzymes for glutathionylating and deglutathionylating proteins are not fully understood although comparison of the reaction mechanism and kinetics with different protein targets have revealed factors that may influence protein binding and glutathione exchange between the enzyme and target. In general, it is believed that not all cysteine residues are equally susceptible to S-glutathionylation in response to the same stimuli. However, no consensuses motif has yet been identified, relating to S-glutathionylation. Several

different contributing factors conferring specificity have been however been pointed out and are discussed below.

Firstly, the accessibility of the thiol group of a given cysteine residue within the protein three-dimensional structure is the primary factor determining if the thiol of this cysteine can undergo S-glutathionylation. The reactivity of the cysteine residues is another determining factor which is dependent upon the adjacent residues or its microenvironment. It has been suggested that if the cysteine is surrounded with positive charged residues, it is more reactive because a P-S^{-} may form through the interaction of the thiol group with the charged residues. As such, a cationic environment can make the –SH group particularly susceptible to S-glutathionylation. Thirdly, the helix-dipole effect has been indicated to contribute to the reactivity of the cysteine residues, which could lower the pKa of cysteine residues. Moreover, cysteine residues form hydrogen bonds with charged residues e.g. Ser or His, selenocysteine residues or cysteines bound with metal ions, e.g. Mg^{2+}, Ca^{2+} or Zn^{2+} are also readily S-glutathionylated (32).

The ability of enzymes such as Grxs, Trxs and sulfiredoxin to catalyse glutathionylation and deglutathionylation, relies on the exposure of the target thiol on the surface of the protein (32). Steric hindrance may prevent the cysteine thiol from interacting with the active site of the enzyme in order to initiate the disulphide exchange (33, 34). As an example of this, although computational modelling predictions using Cyclophilin A place Cys 52 and Cys 161 (out of 4 cysteine residues) on the surface of the protein making them more susceptible to redox modifications, Cys 52 and Cys 62 but not Cys 161 were found to be significantly glutathionylated in T lymphocytes (35). This suggests that target specificity cannot be attributed to solvent exposure alone and is dependent on multiple

(yet to be fully characterized) parameters. Also, the structural conformation of the enzyme itself, likely dictates the ability of the target protein to 'dock' into the crevice accommodating the active site of the enzyme to enable transient physical interactions between the two proteins, enhancing the stability of the protein complex (32, 36).

4.3. 8 GSTs and Glutathionylation

The crystal structure of the Omega glutathione transferase family of proteins is suggestive of a novel role of these proteins in the glutathionylation cycle. Typical of the GST superfamily, GSTO1-1 possesses the typical GST fold comprised of the N-terminal 'thioredoxin' domain which contains the GSH binding site and C-terminal alpha helical domain (37). Most GSTs possess a tyrosine or serine residue at their active site, responsible for catalysing conjugation reactions between GSH and electrophilic substrates. A study by Menon et al. 2013 (1) confirmed for the first time that the protein GST-Omega-1, catalyses protein de/glutathionylation. As mentioned earlier, there is high structural homology between the CLIC proteins and Glutathione-S-Transferase superfamily of enzymes and in particular with the GST-Omega-1 group. Comparison of the two proteins, CLIC1 and GST-omega1 shows they both contain a single cysteine residue (Cys24 in CLIC1 and Cys32 in GSTO Ω1-1) in their active sites and have the ability to form a mixed disulfide bond with glutathione (5, 38-41). On detailed dissection of the crystal structure, the active cysteine (Cys24 in CLIC1 and Cys32 in GSTO Ω1-1) were found to form a disulphide bond with GSH, a feature that is characteristic of the glutaredoxins. Additionally, the GSH binding site is interestingly positioned in a wide crevasse that can potentially accommodate large substrates such as proteins, suggesting potential interactions with molecules apart from GSH (42).

This chapter summarizes and justifies the relevant evidence in support for the hypothesis that members of the CLIC family would also be capable of this de/glutathionylation activity. This information led to the main objective of this study to determine if CLICs proteins have de/glutathionylation activity.

4. 4 Materials and Methods

4.4. 4 Measuring glutathionylation/deglutathionylation activity of CLIC family members

The catalytic glutathionylation / deglutathionylation activity was measured according to the method described by Peltoniemi *et al.* (2006) to evaluate the glutathionylation / deglutathionylation activity of the purified CLICs proteins (43). In this project, the catalytic glutathionylation / deglutathionylation assay was performed for both CLIC wild type proteins and mutant forms of CLIC proteins. This glutathionylation / deglutathionylation assay was performed using an 8-residue designed substrate peptide. The reduced substrate peptide (SQLW**C**LSN), which is used for the glutathionylation assay and the glutathionylated substrate peptide SQLWC[SG]LSN, which is used for the deglutathionylation assay were ordered from the company GenScript (China).

In this assay, each catalytic deglutathionylation assay was performed in triplicate in a 96-well plate (200 µl each well), using the same final concentration (10 µM) of each CLIC protein added to McIlvaine's buffer (2 M sodium hydrogen phosphate, 1 M citric acid, pH 7.0), 1 mM GSH, 50 µM NADPH, 50 nM Glutathione Reductase (GR), 1 mM EDTA, 0-20 µM of the glutathionylated peptide (SQLW**C**[SG]LSN). Fluorescence was recorded at an excitation wavelength 280 nm and an emission wavelength of 356 nm at 25°C using

a BioTek Power Wave TM microplate spectrophotometer. All kinetic data analysis was performed using Microsoft Excel 2010.

For the glutathionylation assay, the assay also was performed in triplicate in a 96-well plate (200 µl each well), containing the same final concentration of 10 µM for each of the CLIC proteins added to McIlvaine's buffer (2 M sodium hydrogen phosphate, 1 M citric acid, pH 7.0), along with GSSG (5 mM), 50 µM NADPH, 50 nM GR, 1 mM EDTA, 0-20 µM of the reduced substrate peptide (SQLWCLSN). Fluorescence was recorded at an excitation wavelength 280 nm and an emission wavelength of 356 nm at 25°C using a BioTek Power Wave TM microplate spectrophotometer. All kinetic data analysis was performed using Microsoft Excel 2010. Background levels determined using the no protein control, were subtracted from the test samples.

4.4. 5 Pre-incubation of CLIC Proteins with Ion Channel Blocker Drug

Stock solutions of 560µM of IAA-94 were freshly prepared by dissolving 0.2 mg/mL of the drug in ethanol with vertexing until a homogeneous solution of the drug in ethanol was achieved. IAA-94 were further diluted to 10 µM in McIlvaine's buffer (2 M sodium hydrogen phosphate, 1 M citric acid, pH 7.0), 10 µM final concentration of CLIC1, 3 and 4 proteins were incubated with 10 µM IAA-94 for 1 hour prior to performing the glutathionylation / deglutathionylation enzyme assay. The glutathionylation/ deglutathionylation enzyme assay was performed as described in the section 4.4.1 above.

4.4. 6 Pre-incubation of CLIC proteins with Cholesterol

50 µl of the cholesterol stock solution (1 mg/ml in chloroform) was added to 200 µl of KCl/Hepes buffer in a glass test tube. The chloroform was removed using N_2 gas for duration of 1 hour. Recombinant CLIC protein (50 µg) was then added to the buffer

solution containing the cholesterol and incubated for an hour on ice. As a control, CLIC proteins were pre-incubated in buffer only without cholesterol. The glutathionylation / deglutathionylation enzyme assay was performed as described in section 4.4.1.

4. 5 Results

4.5. 9 In vitro glutathionylation by CLIC1 protein, CLIC1 mutants and the CLIC-like protein (Exc-4)

The glutathionylating activity of CLIC1 wt, its mutants and CLIC-like protein (Exc-4) were tested using the non-glutathionylated form of the SQLWCLSN peptide, in the presence of high oxidized glutathione (GSSG) concentrations. Glutathionylation by the various CLIC proteins were then measured by monitoring the change in fluorescence, which is emitted by tryptophan. In these reactions as the GSH was added from the neighbouring cysteine, we would expect to see a decrease in fluorescence.

The rates of glutathionylation only increased marginally in the presence of the CLIC1 wt. As expected, CLIC1-Cys24A had minimal to no impact on the reaction rate. Similarly, the mutant forms of CLIC1 (R29A and K37A) and CLIC-like protein (Exc-4) show apparent lower glutathionylation activity when compared to CLIC1 protein under these conditions Figure 4.4. The absolute rate of the glutathionylation activity was low when compared with the deglutathionylation activity of CLIC1 protein (results shown in the subsequent section 4.5.4) and when compared to published deglutathionylation results for GSTO1-1 which showed an approximately 100 fold change in Fluorescence units over a comparable time period of 4-5 minutes in the same assay (1).

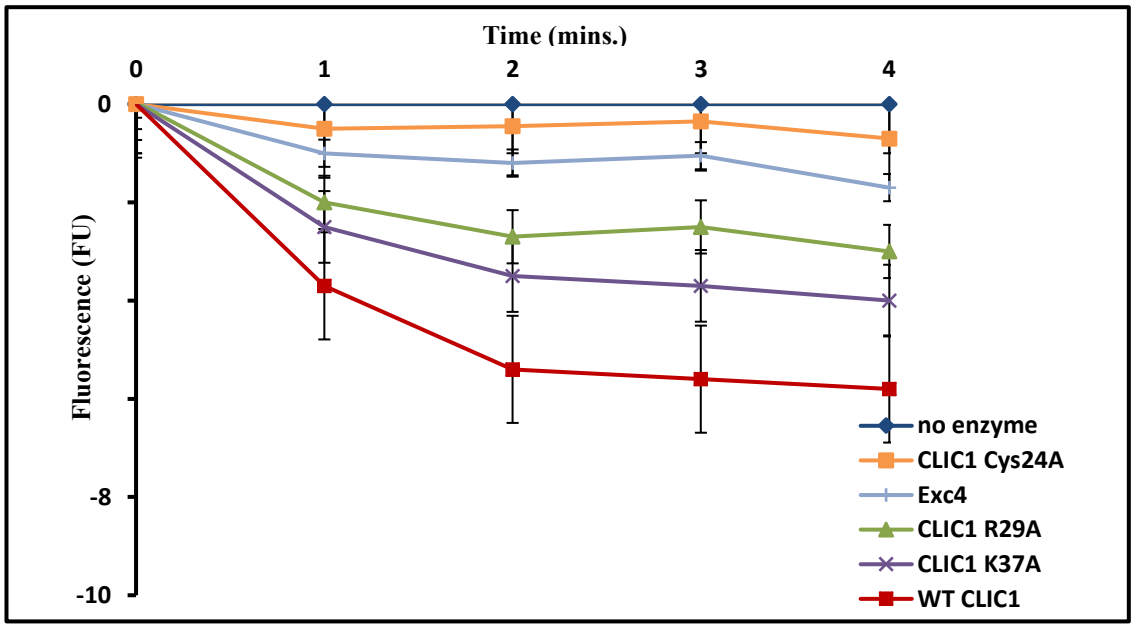

Figure 4. 4 Assaying glutathionylation of a peptide substrate by CLIC1 and CLIC1 mutants. *The change in tryptophan fluorescence indicates glutathionylation of the peptide (SQLWCLSN) by recombinant human CLICs in the presence of GSSG. The results are representative traces from three replicates. Error bars represent the standard deviation of the three independent measurements.*

4.5. 10 In vitro glutathionylation by CLIC3 protein and CLIC3 mutant

CLIC3 protein and its mutant form were assayed in the tryptophan fluorescence quenching assay. As seen in Figure 4.5, the rate of glutathionylation was increased in the presence of the CLIC3 wt, however this was lower than for CLIC1. CLIC3-Cys22,25A as would be expected, showed no change in the fluorescence intensity, indicating no glutathionylation activity.

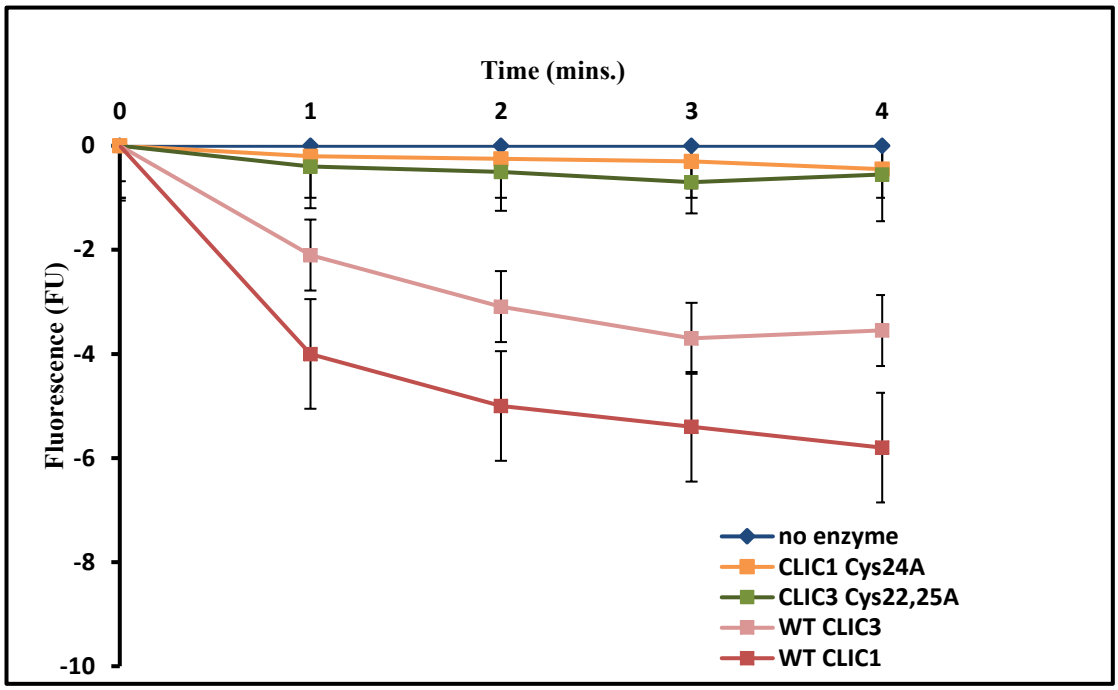

Figure 4. 5 Assaying glutathionylation of a peptide substrate by CLIC1 and CLIC3. *The change in tryptophan fluorescence indicates the rate of peptide (SQLWCLSN) glutathionylation by recombinant human CLICs in the presence of GSSG. The results are representative traces from three replicates. The error bars indicate the standard deviation from three independent readings.*

4.5. 11 In vitro glutathionylation by CLIC4 protein

CLIC4 was also tested in the tryptophan fluorescence quenching assay. As seen in Figure 4.6, CLIC4 wt showed similarly low glutathionylation activity when compared with the CLIC1 wt protein under these conditions, as did CLIC1-Cys24A.

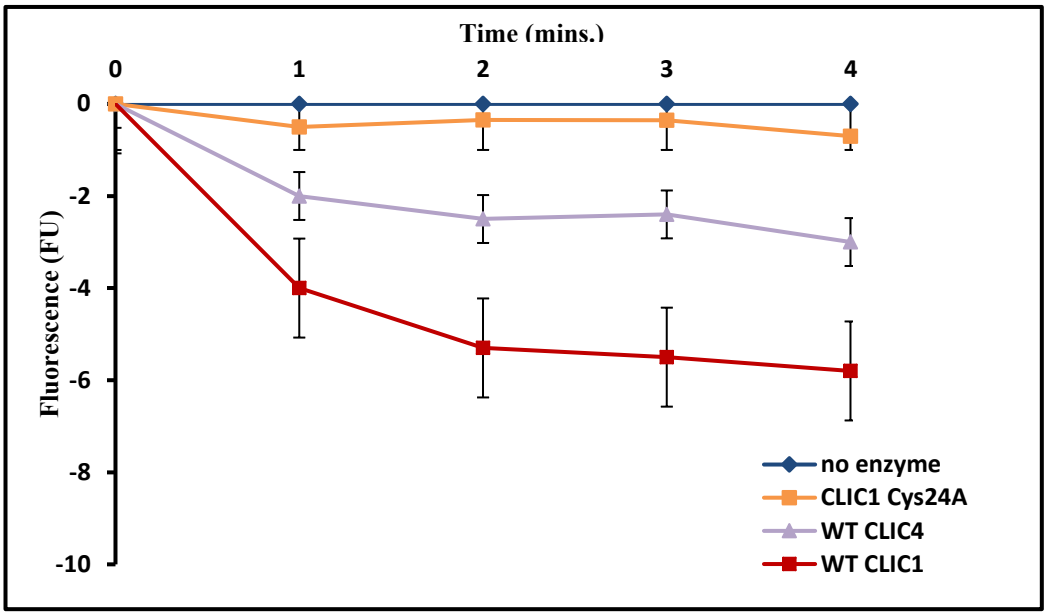

Figure 4. 6 Assaying the glutathionylation activity CLIC1 and CLIC4. *The change in tryptophan fluorescence indicates the rate of peptide (SQLWCLSN) glutathionylation by recombinant human CLICs in the presence of GSSG. The results are representative traces from three replicates. Error bars represents the standard deviation of three independent measurements.*

4.5. 12 In vitro deglutathionylation assay using CLIC1, CLIC1 mutants and the CLIC-like protein (Exc-4)

To determine if members of the CLIC protein family participate in the glutathionylation cycle we adapted a peptide-based tryptophan fluorescence quenching assay that has been previously used to measure the deglutathionylation activity of glutaredoxin (43). A synthetic peptide incorporating a single cysteine residue adjacent to a tryptophan residue (SQLWC-[SG]LSN) was used as the substrate. Deglutathionylation by CLIC proteins were then measured by monitoring the change in fluorescence, which is emitted by tryptophan as the GSH was removed from the neighbouring cysteine. Figure 4.7 shows

the change in fluorescence was dramatically increased in the presence of CLIC1 wt (approx. 200 fold increase over a 3-4 minutes period), but this was not seen with the active site cysteine 24 mutant (Cys24A) which we have previously shown to also lack oxidoreductase activity (31). The mutants R29A and R37A exhibit lower deglutathionylation activity than CLIC1 wt (almost 2-4 fold lower activity). Interestingly, the CLIC-like protein (Exc-4) also showed much lower deglutathionylation activity in the assay compared with CLIC1 wt Figure 4.4.

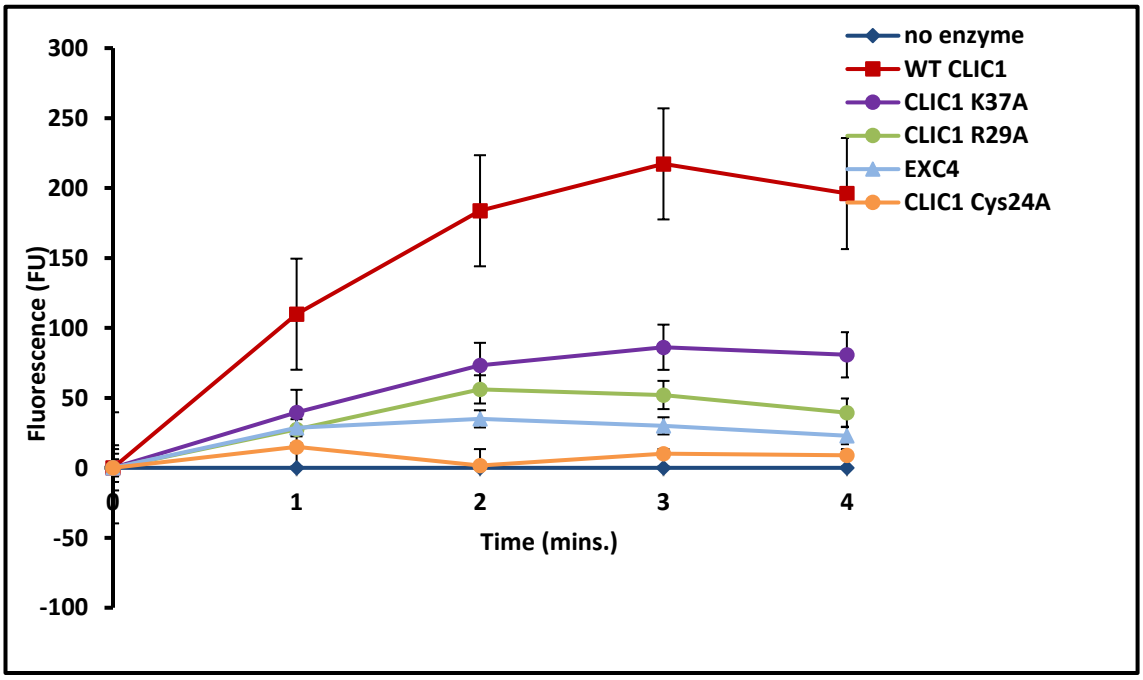

Figure 4. 7 Comparison of CLIC1wt and it's mutants in catalyzing the deglutathionylation of a peptide substrate. *The change in tryptophan fluorescence indicates deglutathionylation of the peptide (SQLWC_[SG]LSN) by recombinant human CLICs in the presence of GSH. The results are representative traces from three replicates. Error bars represents the standard deviation of three independent measurements.*

4.5. 13 In vitro deglutathionylation assay using CLIC3 and CLIC3 mutant

The tryptophan fluorescence quenching assay was employed to characterize the CLIC3 protein deglutathionylation reaction. Figure 4.8 showed that the CLIC3 wt protein catalysed deglutathionylation reaction in a similar manner to the CLIC1 wt protein, albeit at a lower rate (150- fold increase over a 3-4 minutes period). As seen in Figure 4.8 a significant increase in the fluorescence in the presence of CLIC3 protein was evident, but not with the mutant version of CLIC3 (Cys22,25A) where the active site cysteines 22 and 25 where mutated to alanine.

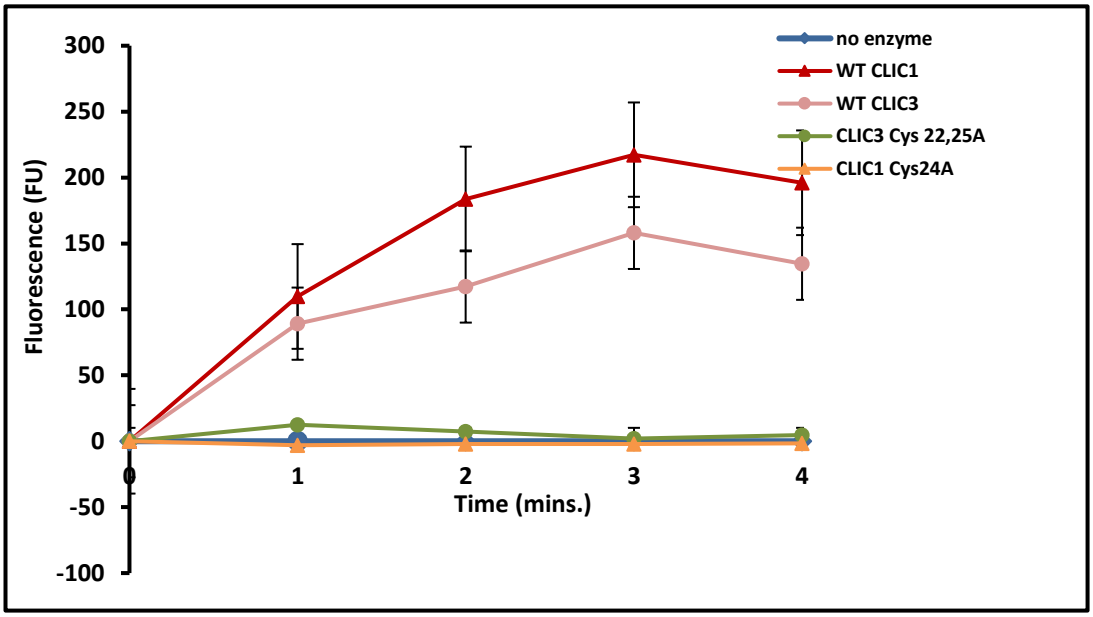

Figure 4. 8 CLIC1 wt and CLIC3 wt catalyze the deglutathionylation of a peptide substrate. *CLIC1 Cys 24A and CLIC3 Cys22,25A showed little to no activity. The change in tryptophan fluorescence indicates the rate of peptide (SQLWC_[SG]LSN) deglutathionylation by recombinant human CLICs in the presence of GSH. The results are representative traces from three replicates. The error bars indicate the standard deviation from three independent readings.*

4.5. 14 In vitro deglutathionylation assay using CLIC4

CLIC4 protein was also assayed in the tryptophan fluorescence quenching assay. As seen in Figure 4.9, CLIC4 wt showed an apparent lower activity when compared with the CLIC1 wt in the deglutathionylation assay, which was similar to the levels seen for CLIC3 (approx. a 150-fold increase over a 3-4 minutes period).

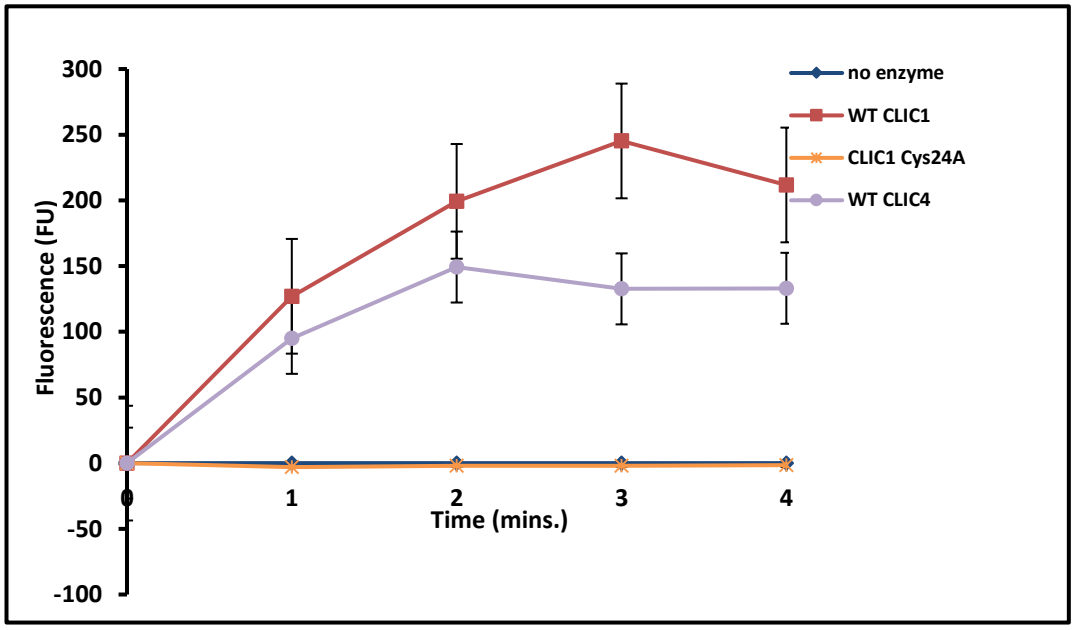

Figure 4. 9 CLIC1 wt and CLIC4 wt catalyze the deglutathionylation of a peptide substrate. *The change in tryptophan fluorescence indicates the rate of peptide (SQLWC_[SG]LSN) deglutathionylation by recombinant human CLICs in the presence of GSH. The results are representative traces from three replicates. Error bars represents the standard deviation of three independent measurements.*

4.5. 15 Effect of chloride ion channel inhibitor drugs on the deglutathionylation activity of CLIC proteins

IAA-94, A9C and DIDS are known chloride ion channel blockers. Studies showed that both IAA-94 and A9C block CLIC1 ion channel activity in cells (44). To determine the effect of IAA-94 on the deglutathionylation activity of CLIC1, CLIC3 and CLIC4; the CLIC proteins were pre-incubated with the IAA-94 for approximately 1 hour. As seen in Figure 4.10, IAA-94 was able to completely block the deglutathionylation activity of CLIC1 wt, CLIC3 wt and CLIC4 wt in the tryptophan fluorescence quenching assay.

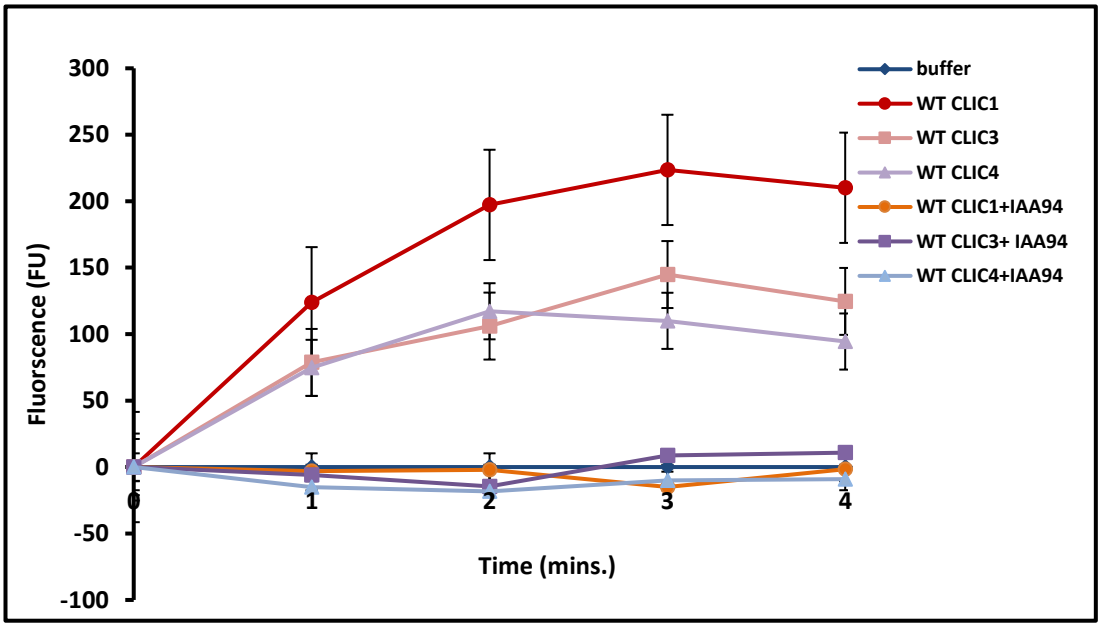

Figure 4. 10 Effect of chloride ion channel inhibitor drugs on the deglutathionylation activity of CLIC1, 3 and 4. *1 uM of CLIC1, 3 and 4 (WT) was incubated with 10 uM IAA-94 for 1-hour prior use of the protein in the assay. The IAA-94 completely blocked the deglutathionylation activity of CLICs. The results are representative traces from three replicates. Error bars represent the standard deviation of three independent measurements.*

4.5. 16 The Effect of Pre-incubation with Cholesterol on the deglutathionylation activity of the CLICs proteins

Impedance spectroscopy studies performed by our group using tethered bilayer membranes (tBLMs) have shown that the presence of cholesterol in these model membranes, regulates the ion channel activity of CLIC1 (45). Comparable results were also obtained with Langmuir film experiments where CLIC1 showed strong preference for associating with or intercalating into single phospholipid or mixed phospholipid monolayers containing cholesterol (46). Impedance spectroscopic studies also revealed that pre-incubation of CLIC1 protein with cholesterol completely diminished ion channel conductivity (45). In order to determine whether cholesterol had a regulatory effect on CLIC protein enzymatic activity, experiments were carried out which involved pre-incubation of CLIC protein samples with cholesterol prior to being assayed with the tryptophan fluorescence quenching assay. Results showed that the cholesterol completely diminished the deglutathionylation activity of the CLIC proteins when it was pre-incubated with cholesterol. As seen in Figure 4.11 the pre-incubation of CLIC1, CLIC3 and CLIC4 wt protein samples with cholesterol greatly inhibited their deglutathionylation activity compared with non-incubated CLIC1, CLIC3 and CLIC4 wt, respectively.

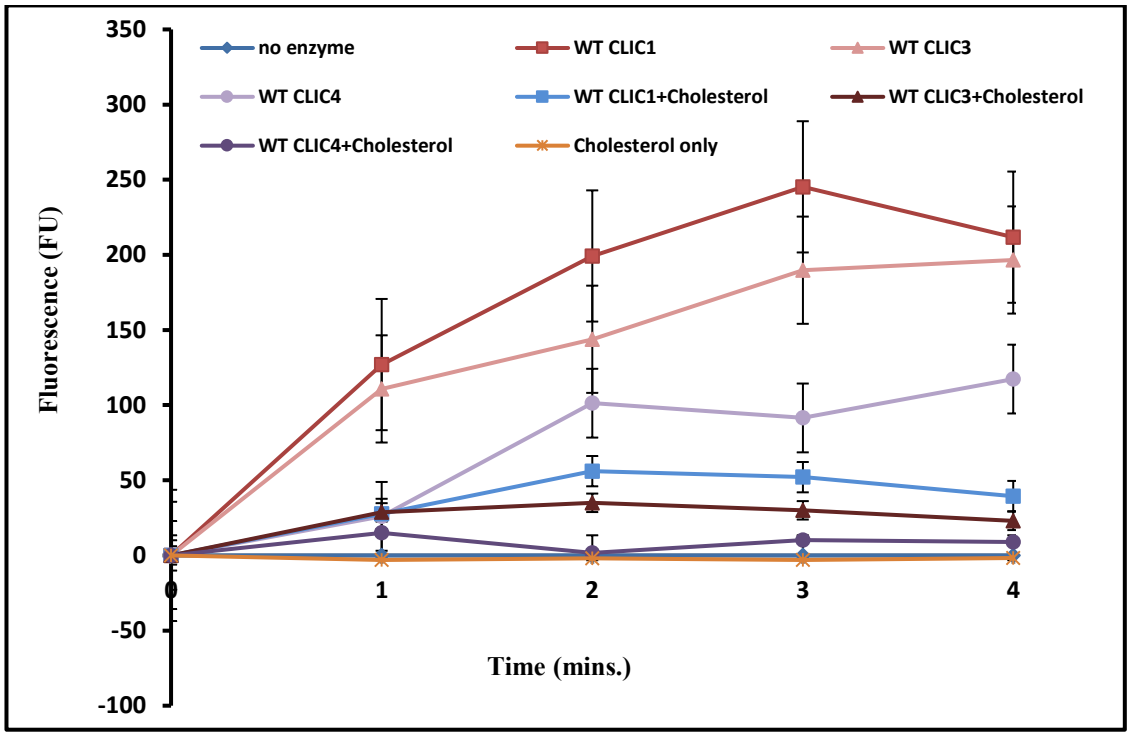

Figure 4. 11 Effect the cholesterol on the deglutathionylation activity of CLICs1, 3 and 4. 1 uM of CLIC1, 3 and 4 (WT) were each incubated with 50 uM of cholesterol for 1 hour prior use of the protein in the assay. The cholesterol greatly diminished the deglutathionylation activity of the CLICs. The results are representative traces from three replicates. *Error bars represent the standard deviation of the three independent measurements.*

4. 6 Discussion

The impact of post-translational modifications such as phosphorylation and glutathionylation on protein structure and function are of great significance. Glutathionylation is relatively less well explored though increasing evidence suggests a growing curiosity in the mechanisms and role of protein thiol modifications with glutathione (3). Glutathionylation is the reversible modification (RTM) of proteins by the addition of a glutathione moiety to thiols exposed on the protein surface. Protein thiols are sensitive to the immediate redox environment and a slight imbalance in redox homeostasis may result in their irreversible oxidation to a sulfonic acid form which may be detrimental to proteins (47). Also, Glutathionylation may be considered as a primary line of defence raised against oxidative stress and hence the reversal of this thiol modification is equally essential for a cell to recover protein function when rescued from an unfavourable environment. Though glutathionylation is strongly associated with oxidative stress, there are several lines of evidence confirming the occurrence of glutathionylation in normal physiological conditions, acting as a switch regulating protein activity (2, 48). The reversal of glutathionylation is largely dependent on catalysts, where the currently best known ones are the glutaredoxins, thioredoxins and sulfiredoxin (3, 49).

The enzymatic rate of glutathionylation/deglutathionylation by glutaredoxin was measured by Peltoniemi *et al.* using an 8 residue long peptide designed to incorporate a single cysteine residue adjacent to tryptophan (SQLW**C**[SG]LSN) (43). Tryptophan, an aromatic amino acid fluoresces when excited at 280 nm. The technique is based on the principle of tryptophan fluorescence quenching. Any change in the immediate environment of tryptophan would alter the fluorescence. For example, when glutaredoxin

glutathionylates the adjacent cysteine residue, tryptophan fluorescence is quenched in a time dependent manner. Alternately, the peptide was artificially glutathionylated to measure the deglutathionylation activity of glutaredoxin. On addition of free GSH to the mix, Grx was found to deglutathionylate the peptide, resulting in a time dependent increase in tryptophan fluorescence. Furthermore, GST Ω1-1 has been shown the ability to deglutathionylate the artificially synthesised peptide (1) in similar manner to the glutaredoxin proteins (43). The technique is well suited to test the activity of potential glutathionylating/deglutathionylating candidate enzymes such as GSTs. Thus, we have characterized the ability of CLIC proteins to catalyse deglutathionylation *in vitro* using the same 8 amino acid artificially synthesised peptide (1, 43).

Our most important overall findings from these studies demonstrate for the first time that the proteins CLIC1, 3 and 4 preferentially catalyse the deglutathionylation over the glutathionylation of peptides / proteins (of which physiological targets are yet to be determined) similar to the glutaredoxins and GSTO1-1 enzymes (1, 43) (Figures 4.7, 4.8 and 4.9). These findings implicate the CLICs proteins as putative participants in the cellular deglutathionylation cycle. Furthermore, our results showed that CLIC1 seems to have a higher deglutathionylation activity compared to CLIC3 and CLIC4, as seen in Figures 4.8 and 4.9. While the nematode CLIC-like protein, exc-4 was found to have significantly lower activity compared to all the tested human CLICs. The studies using the Cysteine mutants confirmed that the redox active Cys24 in CLIC1 and Cys22,25 in CLIC3 are critical for their deglutathionylating activity, Figures 4.7 and 4.8. These findings also support our results from Chapter 2, that showed elimination of the

oxidoreductase enzymatic activity of CLIC3 following the changes of these two Cysteines 22 and 25 to Alanine.

In considering what would account for this difference in the deglutathionylation activity of CLIC3-wt compared with CLIC1-wt, one could speculate that the dithiol active site motif in CLIC3 (CPFC) rather than the monothiol active site motif of CLIC1 (CPFS) is a factor. In support of this is the data published by Menon and Board (2013) (1) who tested CLIC2 - the other dithiol member of CLIC family - in the same assay and found it had little to very low deglutathionylation activity, compared to GSTO1-1.

As to why CLIC4 activity was lower than CLIC1, this is less clear. Given that CLIC4 and CLIC1 contain one cysteine residue (CPFS) in their identical active site and would catalyse the deglutathionylation activity via similar monothiol mechanisms, there are however a number of distinct primary amino acid sequence differences between them. Therefore, it is likely that these surrounding amino acid differences have an impact on their respective active site, either by subtle changes in the structure of the active site or the chemical environment (charged residues, hydrophobicity etc) which could determine specificity of substrate binding and specific targets interactions, under normal physiological conditions.

The low activity demonstrated by the nematode protein Exc-4, could be largely put down to the fact that it does not contain the redox active Cysteine (Cys24 equivalent in CLIC1) but instead has the sequence, DLFC at its active site, with a polar, aspartic acid in place of the redox active Cysteine. In addition, the absence of a proline within this site could also arguably change its reactivity. However, X-ray crystallography studies of Exc-4 confirm that its structure around its active site is similar to the human CLICs despite these

differences (50). Further detailed studies would therefore be needed in order to better define these differences in activity between the various CLICs and CLIC-like proteins.

Further support of the CLIC proteins playing distinct, targeted roles in protein cellular deglutathionylation is via structural studies of CLIC1, that show its GSH binding site is along a groove or open slot (51). This slot or groove could be a binding site for large, extended macromolecules such polypeptides, and it was suggested that the binding of a polypeptide could complete the GSH site and thus enhance its GSH binding affinity, as has also been found with Grx1 (50, 51). Previous structural studies of the glutaredoxins, with their C-terminal active-site cysteine mutated to serine, in mixed disulfide with GSH, implicated a number of conserved residues in the interaction of Grx with the GSH moiety. These mutants include Tyr13, Thr58, Val59, Tyr72, Thr73, and Asp74 in *Escherichia coli* Grx1 and residues in analogous positions in human Grx1 (51). These variants showed a significant decrease in the enzymatic activity, suggesting that all the surrounding active site residues contribute to the GSH interaction site, via changes to the nature of the intermediate formed in the first reaction from Grx-GSH mixed disulfide to the Grx-peptide mixed disulfide (51). Additionally, it has been found that the allelic variants of the GSTO1-1 showed a significant difference in their deglutathionylating activity. The variant D140 exhibited deglutathionylation activity of almost half that of the more frequent (wildtype) A140 isoform (1), while the K208 variant was shown to have approximately five times less activity than the E208 allelic variant (52).

To further investigate the effects of other point mutations located near the active site and investigate their effect on the deglutathionylation activity of CLIC1, we performed deglutathionylation assays on mutant versions of CLIC1 and compared the results to that

of CLIC1-wt. As seen in Figure 4.7, the CLIC1 mutants (R29A and K37A) showed lower deglutathionylation activity compared to CLIC1-wt. These two residues were targeted as they are the only two (positively) charged residues in the entire putative transmembrane domain of CLIC1 (PTMD located between residues 24 – 46 in CLIC1), which also contains the enzymatic active site (CPFS in CLIC1 – commencing at Cys24). Previous studies demonstrated that the mutation of these two charged residues in the PTMD, led to significant changes in CLIC1's biophysical ion channel properties, including a change in the channel open probability (53). In the present study, the deglutathionylation activity of both mutants was reduced to less than half the activity of CLIC1-wt (Figure 4.7). The substitution of the two positive residues, (R29A) and (K37A) to neutral Alanine (Ala), located adjacent to the GSH binding site in CLIC1 protein therefore not surprisingly seem to affect the GSH interaction and hence the binding specificity of CLIC1. This result also coincides with our finding in Chapter 2, where these mutants showed lower oxidoreductase activity in the HEDS assay compared to the CLIC1-wt.

Our group at UTS has undertaken comprehensive studies of the regulatory role of cholesterol and other sterols on the membrane insertion and ion channel activity of CLIC1 (45). Furthermore, our group has previously shown that cholesterol had no clear obvious effect on the protein's enzymatic oxidoreductase activity when tested in the HEDS assay (31). However, in this present study, our results clearly show that pre-incubation of CLIC1, 3 and 4 proteins with cholesterol reduced their deglutathionylation activity, as seen in Figure 4.11. These findings suggest that pre-incubation of the protein samples with cholesterol results in the formation of a relatively stable interaction (pre-complex) between the proteins and the cholesterol in the aqueous phase. Considering that the

CLICs proteins have a GXXXG motif adjacent to the enzyme binding site, one may assume that binding of cholesterol interferes with the protein's enzymatic activity. Analysis of the amino acid sequences done previously by our group (45) has revealed a GXXXG motif in the N-terminal domain that is highly conserved amongst all the human CLIC proteins, several members of the Cholesterol Dependant Cytolysins (examples: listeriolysin, perfringolysin-O) (45) as well as several human membrane proteins (examples: glycophorin-A, ErbB receptor, G-protein coupled receptors) (54).

Previous impedance spectroscopy studies using tBLMs have shown that the ion channel activity of CLIC1 is cholesterol-dependent and pre-incubation of CLIC1 with cholesterol completely abolished its ion channel activity (55). Our group has also demonstrated using Langmuir monolayer film technique, that cholesterol regulates the autonomous insertion of CLIC1 into model membranes and pre-incubating CLIC1 with cholesterol resulted in the formation of a CLIC1-cholesterol pre-complex which prevented the membrane insertion of the pre-incubated CLIC1 protein (56).

Furthermore, our results also show that cholesterol appears to have a different effect on the three different CLIC proteins studied, as shown in Figure 4.11. The deglutathionylation activity of CLIC3 and CLIC4 were more obviously reduced compared to CLIC1. These findings raise questions whether the cholesterol binds with the same affinity to the different types of CLICs proteins, which warrants further investigation to elucidate more details about the structure of the CLICs-cholesterol complex and its apparent regulation of the enzymatic activity of these proteins.

Previous electrophysiological studies by our group and others, has demonstrated that CLIC1 channel activity was blocked by IAA-94 and A9C but not by DIDS (44). Also, our group has shown that the IAA-94 inhibits the oxidoreductase enzymatic activity of the CLIC proteins via the HEDS assay (31). Thus, to evaluate the drugs effect on their deglutathionylation activity in this current assay system, the CLIC proteins were pre-incubated with the IAA-94 drugs for an approximately 1 hour, prior to them being assayed by the tryptophan fluorescence quenching assay. The results showed that the drug IAA-94 was also able to completely block the deglutathionylation activity of CLIC1, CLIC3 and CLIC4 (Figure 4.10).

Furthermore, these findings are consistent with the structural and evolutionary relationship between the GST and CLIC families as IAA-94 is a homologue of ethacrynic acid (57) which is a known inhibitor of the enzymatic activity of a number of GSTs (58) a point also noted on the determination of the structure of CLIC1(5). In fact, the transition of CLIC1 from the soluble form to the integral membrane form is likely to result in a complete disruption of the thioredoxin-like N-terminal domain of the CLIC1 GST fold (5, 59-61). Hence if IAA-94 binds to the soluble form of CLIC1 in the cleft between the N-domain and the C-domain, as is known from the structures of the GST proteins (62, 63) it may be argued that it would be unlikely to bind directly to the integral membrane form as this binding site would no longer exist. Along these lines, we assumed that the drug IAA-94, acts by binding near the active site of the soluble form of CLICs proteins thus inhibiting its deglutathionylating activity and enzymatic activity and consequently it suggests that its channel activity, is under the control of the enzymatic activity of the soluble form of the proteins.

Putative Physiological Targets for the CLIC Proteins Deglutathionylating Activity

The CLICs proteins likely target specific protein substrates just like GSTO, which has been shown to target actin for deglutathionylation (1). The study showed actin was glutathionylated only in the control cells but not in the GSTO1-1 expressing cells, confirming that GSTO1-1 is required for the deglutathionylation of β-actin (1). Similarly, our results from Chapter 2 showed that the CLIC3 protein interacts with TGM2 – therefore it is likely that it is through its deglutathionylation activity that it regulates this protein's activity (64). As such, CLIC3 and other CLICs likely have their own set of specific targets which would be location and condition specific.

Members of the Glutathione transferase family have been well characterized with excellent ability to catalyse the conjugation of glutathione to a range of xenobiotics (14). A relatively new hypothesis proposed previously questioned the possibility of extending GST-catalysed glutathione conjugation reactions to protein substrates (65). So, what could this activity be used for in cells? Though the GSTs have been shown to regulate signal transduction via direct protein-protein interactions (66, 67), GSTP was the first member identified to catalyse protein glutathionylation. The mechanism of glutathionylation by GSTP was shown to be similar to that of typical GSH-conjugation reaction with xenobiotics specifically by lowering the pKa of GSH by the active site tyrosine residue (68, 69). Subsequently, apart from an enzyme's affinity towards a protein target, the susceptibility of the target cysteine residue is also determined by its pKa value which in turn is influenced by the polarity of residues in its immediate environment, either adjacent to the cysteine or in close proximity due to the protein's tertiary structure (70). Furthermore, it has been found that the active site Cysteines surrounded by basic amino

acids such as histidine which could be more susceptible to glutathionylation by lowering the pKa of the cysteine residue (70).

Genetic variation at the CLICs protein locus has previously been linked to the age at onset of Alzheimer's and Parkinson's diseases however a direct functional mechanism has never been identified (71-73). It is reasonable to speculate that differences between family members of CLICs proteins via the deglutathionylation processes could play an important role in the progression of the pathology of different diseases states.

4. 7 Concluding Remarks

Post-translational modifications (PTM) can alter both the functional and structural aspects of proteins. This project investigated the putative biological roles of the CLIC proteins with a focus on one such protein modification known as glutathionylation wherein thiol (-SH) groups in proteins are protected from irreversible oxidation by the addition of glutathione via disulphide bonds. The reaction may be spontaneous or can be catalysed by members of the Glutaredoxin (Grx) family of thioltransferases (28). Therefore, glutathionylation may be seen as a defence mechanism to protect proteins from oxidative stress induced irreversible damage.

In recent years there has been increasing interest in the role of glutathionylation in a wide range of cellular processes including, energy metabolism, signalling and apoptosis, cell cycle regulation, ion channel modulation, calcium homeostasis, regulation of cytoskeletal structures, protein folding and gene regulation, reviewed in (74, 75). As a result of its impact on cellular processes, aberrant glutathionylation has been implicated in the

pathology of a number of clinically significant diseases including Alzheimer's disease, Type 2 diabetes, cystic fibrosis, cataracts and cancer (32, 34, 68, 69, 74).

Because of the increasing recognition of the biological and clinical importance of glutathionylation, a range of techniques have been developed to identify, and locate glutathionylated proteins and to determine the level of glutathionylation in cells.

The results of this chapter provide strong evidences to the important regulatory role of CLICs proteins in the deglutathionylation cycle. A previous study showed that the GSTO1-1 was found to catalyse the deglutathionylation of protein thiols *in vitro* (1). There is a high structural similarity between GSTO1-1, glutaredoxin and CLICs proteins, a thiol transferase catalysing enzyme, and its inability to recognize specific substrates typical of other GSTs, it was clear that its primary function remained to be elucidated. It is however clear that the CLICs proteins also catalyse protein deglutathinylation.

Taken together with observations that members of the CLICs proteins family, which are known to function as ion channels when integrated into membranes, also demonstrate glutaredoxin-like enzymatic activity when found in their soluble form (Chapter 2). This supports an additional role for these proteins in the cellular processes of detoxification and oxidoreduction via the glutathionylation cycle.

4. *8 References*

1. **Menon D, Board PG.** 2013. A role for glutathione transferase Omega 1 (GSTO1-1) in the glutathionylation cycle. Journal of Biological Chemistry **288:**25769-25779.
2. **Wang J, Boja ES, Tan W, Tekle E, Fales HM, English S, Mieyal JJ, Chock PB.** 2001. Reversible glutathionylation regulates actin polymerization in A431 cells. Journal of Biological Chemistry **276:**47763-47766.
3. **Dalle-Donne I, Rossi R, Colombo G, Giustarini D, Milzani A.** 2009. Protein S-glutathionylation: a regulatory device from bacteria to humans. Trends in biochemical sciences **34:**85-96.
4. **Mieyal JJ, Gallogly, M. M., Qanungo, S., Sabens, E. A., & Shelton, M. D. .** 2008. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. . Antioxidants and Redox Signaling, **10** 1941-1988.
5. **Harrop SJ, DeMaere MZ, Fairlie WD, Reztsova T, Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Jankova L, Warton K.** 2001. Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-Å resolution. Journal of Biological Chemistry **276:**44993-45000.
6. **Littler DR, Assaad NN, Harrop SJ, Brown LJ, Pankhurst GJ, Luciani P, Aguilar MI, Mazzanti M, Berryman MA, Breit SN.** 2005. Crystal structure of the soluble form of the redox-regulated chloride ion channel protein CLIC4. FEBS Journal **272:**4996-5007.

7. **Li M, Huang W, Yang Q, Liu X, Wu Q.** 2005. Expression and oxidative stress tolerance studies of glutaredoxin from cyanobacterium Synechocystis sp. PCC 6803 in Escherichia coli. Protein expression and purification **42:**85-91.

8. **Hwang C, Sinskey AJ, Lodish HF.** 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. Science **257:**1496-1502.

9. **Rossjohn J, Polekhina G, Feil SC, Allocati N, Masulli M, Di Ilio C, Parker MW.** 1998. A mixed disulfide bond in bacterial glutathione transferase: functional and evolutionary implications. Structure **6:**721-734.

10. **Cao X, Kambe F, Lu X, Kobayashi N, Ohmori S, Seo H.** 2005. Glutathionylation of two cysteine residues in paired domain regulates DNA binding activity of Pax-8. Journal of Biological Chemistry **280:**25901-25906.

11. **Ghezzi P.** 2005. Oxidoreduction of protein thiols in redox regulation. Biochemical Society Transactions **33:**1378-1381.

12. **Holmgren A.** 1995. Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. Structure **3:**239-243.

13. **Meyer Y, Buchanan BB, Vignols F, Reichheld J-P.** 2009. Thioredoxins and glutaredoxins: unifying elements in redox biology. Annual review of genetics **43:**335-367.

14. **Hayes JD, Flanagan JU, Jowsey IR.** 2005. Glutathione transferases. Annu Rev Pharmacol Toxicol **45:**51-88.

15. **Atkinson HJ, Babbitt PC.** 2009. Glutathione transferases are structural and functional outliers in the thioredoxin fold. Biochemistry **48:**11108-11116.

16. **Oakley AJ.** 2005. Glutathione transferases: new functions. Current opinion in structural biology **15:**716-723.

17. **Dulhunty A, Gage P, Curtis S, Chelvanayagam G, Board P.** 2001. The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. Journal of Biological Chemistry **276:**3319-3323.

18. **Suttorp N, Toepfer W, Roka L.** 1986. Antioxidant defense mechanisms of endothelial cells: glutathione redox cycle versus catalase. American Journal of Physiology-Cell Physiology **251:**C671-C680.

19. **Covarrubias L, Hernández-García D, Schnabel D, Salas-Vidal E, Castro-Obregón S.** 2008. Function of reactive oxygen species during animal development: passive or active? Developmental biology **320:**1-11.

20. **Qanungo S, Starke DW, Pai HV, Mieyal JJ, Nieminen A-L.** 2007. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFκB. Journal of Biological Chemistry **282:**18427-18436.

21. **Owen JB, Butterfield DA.** 2010. Measurement of oxidized/reduced glutathione ratio, p 269-277, Protein Misfolding and Cellular Stress in Disease and Aging. Springer.

22. **Miwa S, Beckman KB, Muller F.** 2008. Oxidative stress in aging: from model systems to human diseases. Springer Science & Business Media.

23. **DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, Mangal D, Kenneth HY, Yeo CJ, Calhoun ES.** 2011. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. Nature **475:**106.

24. **Dalle-Donne I, Giustarini D, Rossi R, Colombo R, Milzani A.** 2003. Reversible S-glutathionylation of Cys374 regulates actin filament formation by inducing structural changes in the actin molecule. Free Radical Biology and Medicine **34:**23-32.

25. **Johansson M, Lundberg M.** 2007. Glutathionylation of beta-actin via a cysteinyl sulfenic acid intermediary. BMC biochemistry **8:**26.

26. **Liao B-C, Hsieh C-W, Lin Y-C, Wung B-S.** 2010. The glutaredoxin/glutathione system modulates NF-κB activity by glutathionylation of p65 in cinnamaldehyde-treated endothelial cells. Toxicological sciences **116:**151-163.

27. **Allen EM, Mieyal JJ.** 2012. Protein-thiol oxidation and cell death: regulatory role of glutaredoxins. Antioxidants & redox signaling **17:**1748-1763.

28. **Shelton MD, Chock PB, Mieyal JJ.** 2005. Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. Antioxidants & redox signaling **7:**348-366.

29. **Lillig CH, Berndt C, Holmgren A.** 2008. Glutaredoxin systems. Biochimica et Biophysica Acta (BBA)-General Subjects **1780:**1304-1317.

30. **Wu G, Fang Y-Z, Yang S, Lupton JR, Turner ND.** 2004. Glutathione metabolism and its implications for health. The Journal of nutrition **134:**489-492.

31. **Al Khamici H, Brown LJ, Hossain KR, Hudson AL, Sinclair-Burton AA, Ng JPM, Daniel EL, Hare JE, Cornell BA, Curmi PM.** 2015. Members of the Chloride Intracellular Ion Channel Protein Family Demonstrate Glutaredoxin-Like Enzymatic Activity. PloS one **10:**e115699.

32. **Dalle-Donne I, Milzani A, Gagliano N, Colombo R, Giustarini D, Rossi R.** 2008. Molecular Mechanisms and Potential Clinical Significance of S - Glutathionylation, vol 10.

33. **Gallogly MM, Starke DW, Mieyal JJ.** 2009. Mechanistic and kinetic details of catalysis of thiol-disulfide exchange by glutaredoxins and potential mechanisms of regulation. Antioxidants & redox signaling **11:**1059-1081.

34. **Shelton MD, Mieyal JJ.** 2008. Regulation by reversible S-glutathionylation: molecular targets implicated in inflammatory diseases. Molecules and cells **25:**332.

35. **Ghezzi P, Casagrande S, Massignan T, Basso M, Bellacchio E, Mollica L, Biasini E, Tonelli R, Eberini I, Gianazza E.** 2006. Redox regulation of cyclophilin A by glutathionylation. Proteomics **6:**817-825.

36. **Gravina SA, Mieyal JJ.** 1993. Thioltransferase is a specific glutathionyl mixed-disulfide oxidoreductase. Biochemistry **32:**3368-3376.

37. **Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV.** 2000. Identification, characterization, and crystal structure of the Omega class glutathione transferases. Journal of Biological Chemistry **275:**24798-24806.

38. **Ashley R.** 2003. Challenging accepted ion channel biology: p64 and the CLIC family of putative intracellular anion channel proteins (Review). Molecular membrane biology **20:**1-11.

39. **García-Sáez AJ, Mingarro I, Pérez-Payá E, Salgado J.** 2004. Membrane-insertion fragments of Bcl-xL, Bax, and Bid. Biochemistry **43:**10930-10943.

40. **Littler DR, Harrop SJ, Fairlie WD, Brown LJ, Pankhurst GJ, Pankhurst S, DeMaere MZ, Campbell TJ, Bauskin AR, Tonini R.** 2004. The intracellular chloride ion channel protein CLIC1 undergoes a redox-controlled structural transition. Journal of Biological Chemistry **279:**9298-9305.

41. **Thuduppathy GR, Craig JW, Kholodenko V, Schon A, Hill RB.** 2006. Evidence that membrane insertion of the cytosolic domain of Bcl-X L is governed by an electrostatic mechanism. Journal of molecular biology **359:**1045-1058.

42. **Whitbread AK, Masoumi A, Tetlow N, Schmuck E, Coggan M, Board PG.** 2005. Characterization of the omega class of glutathione transferases. Methods in enzymology **401:**78-99.

43. **Peltoniemi MJ, Karala A-R, Jurvansuu JK, Kinnula VL, Ruddock LW.** 2006. Insights into Deglutathionylation Reactions DIFFERENT INTERMEDIATES IN THE GLUTAREDOXIN AND PROTEIN DISULFIDE ISOMERASE CATALYZED REACTIONS ARE DEFINED BY THE γ-LINKAGE PRESENT IN GLUTATHIONE. Journal of Biological Chemistry **281:**33107-33114.

44. **Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Warton K, Musgrove EA, Campbell TJ, Breit SN.** 2000. The nuclear chloride ion channel NCC27 is involved in regulation of the cell cycle. The Journal of physiology **529:**541-552.

45. **Valenzuela SM, Alkhamici H, Brown LJ, Almond OC, Goodchild SC, Carne S, Curmi PM, Holt SA, Cornell BA.** 2013. Regulation of the membrane insertion and conductance activity of the metamorphic chloride intracellular channel protein CLIC1 by cholesterol.

46. **Hossain KR, Al Khamici H, Holt SA, Valenzuela SM.** 2016. Cholesterol promotes interaction of the protein CLIC1 with phospholipid monolayers at the air–water interface. Membranes **6:**15.

47. **Lindahl M, Mata-Cabana A, Kieselbach T.** 2011. The disulfide proteome and other reactive cysteine proteomes: analysis and functional significance. Antioxidants & redox signaling **14:**2581-2642.

48. **Bedhomme M, Adamo M, Marchand CH, Couturier J, Rouhier N, Lemaire SD, Zaffagnini M, Trost P.** 2012. Glutathionylation of cytosolic glyceraldehyde-3-phosphate dehydrogenase from the model plant Arabidopsis thaliana is reversed by both glutaredoxins and thioredoxins in vitro. Biochemical Journal **445:**337-347.

49. **Michelet L, Zaffagnini M, Massot V, Keryer E, Vanacker H, Miginiac-Maslow M, Issakidis-Bourguet E, Lemaire SD.** 2006. Thioredoxins, glutaredoxins, and glutathionylation: new crosstalks to explore. Photosynthesis Research **89:**225-245.

50. **Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, Valenzuela SM, Mazzanti M, Brown LJ, Breit SN.** 2010. The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? FEBS letters **584:**2093-2101.

51. **Saaranen MJ, Salo KE, Latva-Ranta MK, Kinnula VL, Ruddock LW.** 2009. The C-terminal active site cysteine of Escherichia coli glutaredoxin 1 determines the glutathione specificity of the second step of peptide deglutathionylation. Antioxidants & redox signaling **11:**1819-1828.

52. **Zhou H, Brock J, Casarotto MG, Oakley AJ, Board PG.** 2011. Novel folding and stability defects cause a deficiency of human glutathione transferase omega 1. Journal of Biological Chemistry **286:**4271-4279.

53. **Averaimo S, Abeti R, Savalli N, Brown LJ, Curmi PM, Breit SN, Mazzanti M.** 2013. Point mutations in the transmembrane region of the CLIC1 ion channel selectively modify its biophysical properties. PloS one **8:**e74523.

54. **Prakash A, Janosi L, Doxastakis M.** 2011. GxxxG motifs, phenylalanine, and cholesterol guide the self-association of transmembrane domains of ErbB2 receptors. Biophysical journal **101:**1949-1958.

55. **Shinzawa-Itoh K, Aoyama H, Muramoto K, Terada H, Kurauchi T, Tadehara Y, Yamasaki A, Sugimura T, Kurono S, Tsujimoto K.** 2007. Structures and physiological roles of 13 integral lipids of bovine heart cytochrome c oxidase. The EMBO journal **26:**1713-1725.

56. **Hossain KR.** 2016. A study of the spontaneous membrane insertion of chloride intracellular ion channel protein CLIC1 into model lipid membranes.

57. **Landry DW, Akabas MH, Redhead C, Edelman A, Cragoe EJ, Al-Awqati Q.** 1989. Purification and reconstitution of chloride channels from kidney and trachea. Science **244:**1469-1472.

58. **Ploemen J, Ommen BV, Bogaards J, Van Bladeren P.** 1993. Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione S-transferases. Xenobiotica **23:**913-923.

59. **Goodchild SC, Howell MW, Cordina NM, Littler DR, Breit SN, Curmi PM, Brown LJ.** 2009. Oxidation promotes insertion of the CLIC1 chloride

intracellular channel into the membrane. European Biophysics Journal **39:**129-138.

60. **Goodchild SC, Howell MW, Littler DR, Mandyam RA, Sale KL, Mazzanti M, Breit SN, Curmi PM, Brown LJ.** 2010. Metamorphic response of the CLIC1 chloride intracellular ion channel protein upon membrane interaction. Biochemistry **49:**5278-5289.

61. **Singh H.** 2010. Two decades with dimorphic chloride intracellular channels (CLICs). FEBS letters **584:**2112-2121.

62. **Cameron AD, Sinning I, L'Hermite G, Olin B, Board PG, Mannervik B, Jones TA.** 1995. Structural analysis of human alpha-class glutathione transferase A1-1 in the apo-form and in complexes with ethacrynic acid and its glutathione conjugate. Structure **3:**717-727.

63. **Oakley AJ, Rossjohn J, Lo Bello M, Caccuri AM, Federici G, Parker MW.** 1997. The three-dimensional structure of the human Pi class glutathione transferase P1-1 in complex with the inhibitor ethacrynic acid and its glutathione conjugate. Biochemistry **36:**576-585.

64. **Hernandez-Fernaud JR, Ruengeler E, Casazza A, Neilson LJ, Pulleine E, Santi A, Ismail S, Lilla S, Dhayade S, MacPherson IR.** 2017. Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity. Nature communications **8:**14206.

65. **Listowsky I.** 2005. Proposed intracellular regulatory functions of glutathione transferases by recognition and binding to S-glutathiolated proteins. The Journal of peptide research **65:**42-46.

66. **Laborde E.** 2010. Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. Cell death and differentiation **17:**1373.

67. **Liu D, Hewawasam R, Pace SM, Gallant EM, Casarotto MG, Dulhunty AF, Board PG.** 2009. Dissection of the inhibition of cardiac ryanodine receptors by human glutathione transferase GSTM2-2. Biochemical pharmacology **77:**1181-1193.

68. **Tew KD, Townsend DM.** 2011. Regulatory functions of glutathione S-transferase P1-1 unrelated to detoxification. Drug metabolism reviews **43:**179-193.

69. **Townsend DM, Manevich Y, He L, Hutchens S, Pazoles CJ, Tew KD.** 2009. Novel role for glutathione S-transferase π regulator of protein S-glutathionylation following oxidative and nitrosative stress. Journal of biological chemistry **284:**436-445.

70. **Grek CL, Zhang J, Manevich Y, Townsend DM, Tew KD.** 2013. Causes and consequences of cysteine S-glutathionylation. Journal of Biological Chemistry **288:**26497-26504.

71. **Novarino G, Fabrizi C, Tonini R, Denti MA, Malchiodi-Albedi F, Lauro GM, Sacchetti B, Paradisi S, Ferroni A, Curmi PM.** 2004. Involvement of the intracellular ion channel CLIC1 in microglia-mediated β-amyloid-induced neurotoxicity. The Journal of neuroscience **24:**5322-5330.

72. **Skaper SD, Facci L, Giusti P.** 2013. Intracellular ion channel CLIC1: involvement in microglia-mediated β-amyloid peptide (1-42) neurotoxicity. Neurochemical research **38:**1801-1808.

73. **Thei L, Imm J, Kaisis E, Dallas ML, Kerrigan TL.** 2018. Microglia in Alzheimer's Disease: a role for Ion Channels. Frontiers in neuroscience **12**.

74. **Cooper AJ, Pinto JT, Callery PS.** 2011. Reversible and irreversible protein glutathionylation: biological and clinical aspects. Expert opinion on drug metabolism & toxicology **7:**891-910.

75. **De Luca A, Moroni N, Serafino A, Primavera A, Pastore A, Pedersen JZ, Petruzzelli R, Farrace MG, Pierimarchi P, Moroni G.** 2011. Treatment of doxorubicin-resistant MCF7/Dx cells with nitric oxide causes histone glutathionylation and reversal of drug resistance. Biochemical Journal **440:**175-183.

Chapter 5

Key findings and Conclusions

Chapter 5

5.1 Key findings and Conclusions

This PhD project has extended our knowledge around the functional activity of members of the CLIC protein family. In particular CLIC1, CLIC3 and CLIC4 were studied. These proteins are well known to function as ion channels when integrated into membranes, but they also demonstrate glutaredoxin-like, glutathione-dependent oxidoreductase enzymatic activity, with this being the first study to demonstrate this function for CLIC3 (Chapter 2). The CLICs resemble the activity of the GST-Ω and the monothiol glutaredoxin proteins. In addition, in a broader sense, this project also aimed to demonstrate the ability of the CLIC proteins to act as oxidoreductase enzymes, providing a protective role against oxidative stress within cells. For this we were able to show that they serve to protect cells against oxidative damage and circumvent oxidative stress within cells via an *in-situ* cell model system (Chapter 3). Furthermore, this study identified a novel capability of the CLIC proteins in the deglutathionylation of protein targets (Chapter 4).

Previous studies by our lab group have shown that the soluble CLIC protein members CLIC1, CLIC2 and CLIC4, are able to function as enzymes that resemble the activity of the GST-Ω and the monothiol glutaredoxin proteins (1). Our group also demonstrated that the residue Cysteine 24 plays a critical role in the enzymatic activity of CLIC1, via mutation of Cys24 to either serine or alanine, which caused the full elimination of the enzymatic activity of CLIC1 in the HEDS enzyme assay.

The current study results in Chapter 2 have shown that the soluble CLIC3 protein exhibits glutaredoxin-like thiol transferase activity using the chemical substrate 2-hydroxyethyl

disulphide (HEDS) in a similar manner to the CLIC1 protein (Figure 2.12). Interestingly, mutating both Cys 22 and 25 to Alanine in CLIC3 resulted in complete inhibition of its enzymatic activity, while mutating only Cys22 to alanine, resulted in a substantial decrease in its enzymatic activity but not total inhibition. This confirms that both Cys22 and Cys25 residues in CLIC3 are essential for its oxidoreductase enzymatic activity and thus it's characterisation as a dithiol active G-site enzyme, Figure 2.17.

Our collaborators at the Beatson Institute for Cancer Research - Glasgow University, showed that the CLIC3 protein is secreted from Cancer Associated Fibroblasts as well as from MDA-MB-231 breast cancer cells (2) and it has previously been shown to be localised to late endosomal compartments in cells (3). Interestingly, CLIC3 was further identified as a protein that was expressed at significantly higher levels in Cancer Associated Fibroblasts than Normal Fibroblasts. Also, the team in Glasgow found an additional secreted protein whose levels increased in parallel with CLIC3; this was transglutaminase 2 (TGM2). Given our discovery describing several CLIC member's thioredoxin activity (1), and studies indicating that thioredoxin activates TGM2 (4), jointly our teams collaborated to complete studies proving that CLIC3 has oxidoreductase activity and thus could reduce the disulfide bonds of the enzyme TGM2 and hence regulate its enzymatic activity. This information has led to the discovery that extracellular secreted CLIC3, uses its oxidoreductase activity to drive cancer progression, cancer cell invasiveness and angiogenesis via a TGM2-dependent process. Because of CLIC3's ability to drive these TGM2-dependent invasive processes requires 'active site' cysteine residues in CLIC3' thioredoxin fold, it was proposed that the role of extracellular CLIC3 acts via a redox mechanism to influence TGM2 function to promote integrin-dependent cell invasion. Considering the oxidoreductase activity, CLIC3 likely

activates TGM2's enzymatic activity via a thioredoxin-like mechanism, which leads to increased ECM stiffness followed by integrin activation and signaling. This corresponds with data showing that CLIC3 and TGM2 are upregulated in cancer. In addition, TGM2 has been shown to be upregulated in several cancers such as pancreatic adenocarcinoma (5), breast carcinoma (6) and ovarian carcinoma (7). In addition, CLIC3 upregulation has been shown to lead to a poorer survival in pancreatic and ovarian adenocarcinoma patients (3).

It is becoming increasingly clear that the CLIC proteins are emerging as important players in the cancer field. The following points are included in support of this idea. There have now been several reports connecting CLIC family members to carcinomas *in vivo* and indicating their potential suitability to serve as cancer biomarkers. In humans, members of the CLIC family have been linked to nasopharyngeal carcinoma, gastric cancer, hepatocarcinoma, colorectal cancer, gallbladder carcinoma, ovarian and breast cancer (8-10) (further details covering the role of the CLIC proteins in cancers were also included in Chapter 1). Interestingly, CLIC1 has been observed to be secreted by cancer cells and to be detectable in serum and plasma samples of cancer patients (11, 12). CLIC4 has been found to drive tumour cell growth through alterations in the transforming growth factor (TGF-β) signaling within stromal cells (13), while CLIC3 increases invasiveness by driving integrin recycling (3). Several studies also pointed to highly expression of the CLIC proteins in tumours (12-15). It now remains to determine the precise mechanisms by which the CLIC proteins perform these activities in cancer and to determine what unique activities are performed by each CLIC member. As such, our studies provide important new clues on the functional activities of the CLIC proteins as enzymes, allowing further studies on how they are regulated in normal versus cancer cells.

Structural studies have identified that the CLIC proteins can exist in two different conformations in cells; a soluble globular form comprising a GST-fold, that possesses thioreductase activity; and another confirmation displaying amphiphatic helical regions, capable of inserting into cell membranes, which enables Cl- transport across membranes. However, it is now clear they are also found as extracellular secreted proteins. One could therefore speculate that this ability of the CLICs to switch between the soluble globular and the membrane-inserted confirmation, might also serve as a mechanism via which CLIC proteins permeate the plasma membrane and leave or are secreted by cells (16, 17). Furthermore, their presence on extracellular vesicles such as microparticles and exosomes is also another new location indicating potential roles in cell-cell communication, in addition to their functions ranging from Cl- channels, to molecular scaffolds, to functioning as oxidoreductases enzymes (18).

One of the most exciting findings from this PhD study was demonstrating that the CLIC proteins help to maintain cellular hemostasis and participate in crucial roles in a cell's defense against oxidative stress in the presence of oxidants. Over exposure of proteins to ROS can result in the irreversible oxidation of protein thiols and subsequent loss of activity and proteasomal degradation. Chapter 3 of this thesis describes the successful demonstration that CLIC1 and to a lesser extent CLIC3, could act as cell protective proteins, possessing cellular antioxidant activity. In our experimental model we observed significantly higher tolerance to different concentrations of the oxidant H_2O_2 by bacterial *E. coli* cells expressing the protein CLIC1 in comparison to the respective control bacterial *E. coli* cells. Furthermore, the mutant form of the CLIC1 protein lacking the critical cysteine24 residue in the enzymatic active site

did not provide this same protection. Further experiments are now required to elucidate the precise mechanisms by which CLIC proteins provide this cellular antioxidant activity and also demonstrate this in a eukaryotic cell system. However, it is not unreasonable to assume that the CLIC proteins may either be reducing or deglutathionylating protein cysteines that become glutathionylated and thus protecting them from irreversible oxidation and / or restoring their activity. However, this study demonstrates for the first time the *direct* cellular antioxidant activity by members of the CLIC protein family. It also strongly points to CLIC1 likely acting as a stress response protein inside human cells and further supports classification of these proteins as moonlighting proteins, with two distinct functions – membrane ion channels and antioxidant oxidoreductase enzymes.

The focus of Chapter 4 of this thesis was de/glutathionylation which is mainly considered as an oxidative stress triggered protein modification. Previous studies provide evidence indicating that some proteins can be actively deglutathionylated in response to oxidative stress (19). Deglutathionylation regulates protein activity in two ways, firstly by indirectly changing protein charge and redox state and secondly by directly binding to proteins and forming disulphide bonds (20). This reaction is known to be catalysed by members of the Glutaredoxin (Grx), Glutathione transferase Pi (GSTP), Thioredoxin (Trx), Protein disulphide isomerase (PDI), Sulfiredoxin (19).

Furthermore, glutathionylation has been shown to have an important role on the regulation of the cell cycle, apoptosis and inflammation (21-23), as well as the contractile activity of actin and the phosphatase activity of Protein Tyrosine Phosphatase1B (24, 25). Thus, the mechanisms and catalytic mediators of the glutathionylation cycle are of great interest under both oxidative and non-oxidative conditions. Since glutathionylation has also been

implicated in the pathology of Alzheimer's disease, Parkinson's disease, COPD and inflammation. Therefore, it is suggested here that the CLIC proteins glutathionylation and/or deglutathionylation activity could be a common factor in these pathologies.

In this PhD study, CLIC proteins were shown to preferentially catalyse the deglutathionylation of protein thiols *in vitro* using tryptophan fluorescence quenching assay (26) (27). Our results in Chapter 4 also showed that the CLIC1-wt and CLIC3-wt, exhibit high deglutathionylation activity, however their corresponding mutant forms CLIC1-Cys24S and CLIC3-Cys22,25A, lacked this activity, thus confirming the critical role of the active site cysteines in this process. It is important to note that the GSH-binding site in CLICs proteins lies in a relatively open crevice that is large enough to fit interacting proteins. This would raise the possibility that the specificity of CLICs proteins towards protein targets arises from the physical (transient) association of the protein target with CLICs proteins, thereby allowing the glutathionylated residue to bind with the GSH-site and transfer the glutathione moiety to form a disulphide with the active Cysteine residue.

Glutathionylation and deglutathionylation of proteins has previously been predominantly attributed to the Grx family thioltransferases (26), amongst others, and more recently also to Glutathione transferase Omega 1 (GSTO1-1) (27). The current results add the CLIC family members as novel proteins to the list of enzymes catalysing glutathionylation cycle reactions.

5.2 Future directions

In this thesis, we have identified several characteristics of the protein CLIC3. We have determined that CLIC3 functions as oxidoreductase enzyme *in vitro* and now as we have shown, CLICs dependent glutathionylation of target cellular proteins can be determined using cultured cell lines overexpressing CLIC proteins. Additionally, the new discovery that the CLICs proteins have the functional ability to act as general antioxidants and oxidoreductase enzymes which suggests a role for them as cell protective proteins. This is also supported by the structural evidences that the CLICs proteins have a high level of structural and functional similarity with the GST-Ω, Grxs and Trxs with the latter 3 groups have well-known oxidoreductase class of enzymes. Therefore, the CLIC protein family should be included as an additional group within this oxidoreductase enzyme superfamily.

Furthermore, the study done by our collaborators at the Beatson institute for cancer research/ Glasgow university/ UK have shown that the CLIC3 protein under reducing conditions, the possibility to interact with TGM2 and to lead to TGM2 dependent ECM remodeling and tumour cell invasion. Although evidence that these TGM2-dependent functions of CLIC3 are sensitive to the redox environment and require CLIC3's 'active site' cysteines (which suggests a role for CLIC3's oxidoreductase activity), further studies are needed to identify and understand the mechanistic details of the interplay between extracellular CLIC3 and TGM2. For instance, the possibility that CLIC3 affects proteins other than TGM2 to drive tumour cell invasion must be considered. Moreover, a possible unconventional mechanism through which CLIC3 is secreted from CAFs needs to be identified and characterized. In addition, determining the degree to which CLIC3 is secreted by stromal and tumour cells *in vivo* and the relevance that this process has to the progression of cancer in humans.

Our results in Chapter 2 showed that the two positively charged residues: Arginine 29 (Arg29) and lysine 37 (Lys37) located in the putative transmembrane region of CLIC1 protein (60), and which are also arranged in close vicinity to the enzymatic active site (see figure 2.13 in Chapter 2), are likely to have a key role in the enzymatic functional activity of CLIC1. These same two residues have previously been shown to regulate the ion channel activity of CLIC1 (60). Further studies are therefore needed to identify other key residues located near the enzymatic active site of CLIC1 protein. Additionally, further mutagenesis experiments are needed to substitute other residues in different CLIC proteins, like CLIC2, CLIC3 and CLIC4 to identify the importance of these residues on the functional oxidoreductase enzymatic activity of these other CLIC proteins and to discern differences between the family members that may contribute to distinct functions by each family member.

In this thesis we also demonstrated for the first time the novel ability of CLIC proteins in the glutathionylation/deglutathionylation of proteins. Our findings in chapter 4 demonstrated CLIC1, 3 and 4 proteins have significant deglutathionylation activity with a model peptide substrate. Given that our degluathionylation activity studies was done *in vitro*, further studies are needed to investigate this activity *in vivo* and using culture cell lines. Considering that the CLIC proteins participate in the deglutathionylation cycle it now remains to identify specific protein targets and if there are distinct targets for each member of the CLIC family. This could help to provide a significant understanding about the role of CLICs proteins in the oxidoreduction reactions and as post-translational regulators of target proteins via their deglutathionylation activity.

These new discoveries strengthen our key hypotheses and support the concept that the CLICs proteins have a significant and remarkable functions in cells. They also provide novel insights about functional and structural properties of this family. The six vertebrate CLIC proteins are believed to have arisen via gene duplication from an ancient chordate and are highly conserved across species throughout evolution (18). Given all of this information, we are only now coming to appreciate their importance, with this current study adding significant new information supporting that these proteins are significant key players in normal cell homeostasis and will likely be revealed as key players in a variety of pathologies.

5.3 References

1. **Al Khamici H, Brown LJ, Hossain KR, Hudson AL, Sinclair-Burton AA, Ng JPM, Daniel EL, Hare JE, Cornell BA, Curmi PM.** 2015. Members of the Chloride Intracellular Ion Channel Protein Family Demonstrate Glutaredoxin-Like Enzymatic Activity. PloS one **10:**e115699.
2. **Hernandez-Fernaud JR, Ruengeler E, Casazza A, Neilson LJ, Pulleine E, Santi A, Ismail S, Lilla S, Dhayade S, MacPherson IR.** 2017. Secreted CLIC3 drives cancer progression through its glutathione-dependent oxidoreductase activity. Nature communications **8:**14206.
3. **Dozynkiewicz MA, Jamieson NB, MacPherson I, Grindlay J, van den Berghe PV, von Thun A, Morton JP, Gourley C, Timpson P, Nixon C.** 2012. Rab25 and

CLIC3 collaborate to promote integrin recycling from late endosomes/lysosomes and drive cancer progression. Developmental cell **22:**131-145.

4. **Jin X, Stamnaes J, Klöck C, DiRaimondo TR, Sollid LM, Khosla C.** 2011. Activation of extracellular transglutaminase 2 by thioredoxin. Journal of Biological Chemistry**:**jbc. M111. 287490.

5. **Verma A, Wang H, Manavathi B, Fok JY, Mann AP, Kumar R, Mehta K.** 2006. Increased expression of tissue transglutaminase in pancreatic ductal adenocarcinoma and its implications in drug resistance and metastasis. Cancer research **66:**10525-10533.

6. **Mehta K, Fok J, Miller FR, Koul D, Sahin AA.** 2004. Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. Clinical cancer research **10:**8068-8076.

7. **Hwang JY, Mangala LS, Fok JY, Lin YG, Merritt WM, Spannuth WA, Nick AM, Fiterman DJ, Vivas-Mejia PE, Deavers MT.** 2008. Clinical and biological significance of tissue transglutaminase in ovarian carcinoma. Cancer research **68:**5849-5858.

8. **Chen CD, Wang CS, Huang YH, Chien KY, Liang Y, Chen WJ, Lin KH.** 2007. Overexpression of CLIC1 in human gastric carcinoma and its clinicopathological significance. Proteomics **7:**155-167.

9. **Petrova DT, Asif AR, Armstrong VW, Dimova I, Toshev S, Yaramov N, Oellerich M, Toncheva D.** 2008. Expression of chloride intracellular channel protein 1 (CLIC1) and tumor protein D52 (TPD52) as potential biomarkers for colorectal cancer. Clinical biochemistry **41:**1224-1236.

10. **Wang J-W, Peng S-Y, Li J-T, Wang Y, Zhang Z-P, Cheng Y, Cheng D-Q, Weng W-H, Wu X-S, Fei X-Z.** 2009. Identification of metastasis-associated proteins involved in gallbladder carcinoma metastasis by proteomic analysis and functional exploration of chloride intracellular channel 1. Cancer letters **281:**71-81.

11. **Chang Y-H, Wu C-C, Chang K-P, Yu J-S, Chang Y-C, Liao P-C.** 2009. Cell secretome analysis using hollow fiber culture system leads to the discovery of CLIC1 protein as a novel plasma marker for nasopharyngeal carcinoma. Journal of proteome research **8:**5465-5474.

12. **Tang H-Y, Beer LA, Tanyi JL, Zhang R, Liu Q, Speicher DW.** 2013. Protein isoform-specific validation defines multiple chloride intracellular channel and tropomyosin isoforms as serological biomarkers of ovarian cancer. Journal of proteomics **89:**165-178.

13. **Shukla A, Edwards R, Yang Y, Hahn A, Folkers K, Ding J, Padmakumar V, Cataisson C, Suh K, Yuspa S.** 2014. CLIC4 regulates TGF-β-dependent myofibroblast differentiation to produce a cancer stroma. Oncogene **33:**842.

14. **Deng Y-J, Tang N, Liu C, Zhang J-Y, An S-L, Peng Y-L, Ma L-L, Li G-Q, Jiang Q, Hu C-T.** 2014. CLIC4, ERp29, and Smac/DIABLO derived from metastatic cancer stem–like cells stratify prognostic risks of colorectal cancer. Clinical Cancer Research **20:**3809-3817.

15. **Ulmasov B, Bruno J, Gordon N, Hartnett ME, Edwards JC.** 2009. Chloride intracellular channel protein-4 functions in angiogenesis by supporting acidification of vacuoles along the intracellular tubulogenic pathway. The American journal of pathology **174:**1084-1096.

16. **Littler DR, Assaad NN, Harrop SJ, Brown LJ, Pankhurst GJ, Luciani P, Aguilar MI, Mazzanti M, Berryman MA, Breit SN.** 2005. Crystal structure of the soluble form of the redox-regulated chloride ion channel protein CLIC4. FEBS Journal **272:**4996-5007.

17. **Littler DR, Harrop SJ, Fairlie WD, Brown LJ, Pankhurst GJ, Pankhurst S, DeMaere MZ, Campbell TJ, Bauskin AR, Tonini R.** 2004. The intracellular chloride ion channel protein CLIC1 undergoes a redox-controlled structural transition. Journal of Biological Chemistry **279:**9298-9305.

18. **Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, Valenzuela SM, Mazzanti M, Brown LJ, Breit SN.** 2010. The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? FEBS letters **584:**2093-2101.

19. **Liao B-C, Hsieh C-W, Lin Y-C, Wung B-S.** 2010. The glutaredoxin/glutathione system modulates NF-κB activity by glutathionylation of p65 in cinnamaldehyde-treated endothelial cells. Toxicological sciences **116:**151-163.

20. **Dominko K, Đikić D.** 2018. Glutathionylation: a regulatory role of glutathione in physiological processes. Archives of Industrial Hygiene and Toxicology **69:**1-24.

21. **Shelton MD, Mieyal JJ.** 2008. Regulation by reversible S-glutathionylation: molecular targets implicated in inflammatory diseases. Molecules and cells **25:**332.

22. **Townsend DM, Manevich Y, He L, Hutchens S, Pazoles CJ, Tew KD.** 2009. Novel role for glutathione S-transferase π regulator of protein S-glutathionylation following oxidative and nitrosative stress. Journal of biological chemistry **284:**436-445.

23. **Cooper AJ, Pinto JT, Callery PS.** 2011. Reversible and irreversible protein glutathionylation: biological and clinical aspects. Expert opinion on drug metabolism & toxicology **7:**891-910.

24. **Wang J, Boja ES, Tan W, Tekle E, Fales HM, English S, Mieyal JJ, Chock PB.** 2001. Reversible glutathionylation regulates actin polymerization in A431 cells. Journal of Biological Chemistry **276:**47763-47766.

25. **Findlay VJ, Townsend DM, Morris TE, Fraser JP, He L, Tew KD.** 2006. A novel role for human sulfiredoxin in the reversal of glutathionylation. Cancer research **66:**6800-6806.

26. **Peltoniemi MJ, Karala A-R, Jurvansuu JK, Kinnula VL, Ruddock LW.** 2006. Insights into Deglutathionylation Reactions DIFFERENT INTERMEDIATES IN THE GLUTAREDOXIN AND PROTEIN DISULFIDE ISOMERASE CATALYZED REACTIONS ARE DEFINED BY THE γ-LINKAGE PRESENT IN GLUTATHIONE. Journal of Biological Chemistry **281:**33107-33114.

27. **Menon D, Board PG.** 2013. A role for glutathione transferase Omega 1 (GSTO1-1) in the glutathionylation cycle. Journal of Biological Chemistry **288:**25769-25779.